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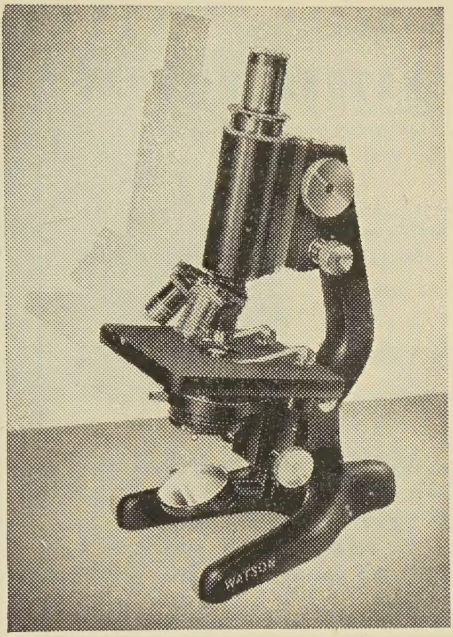

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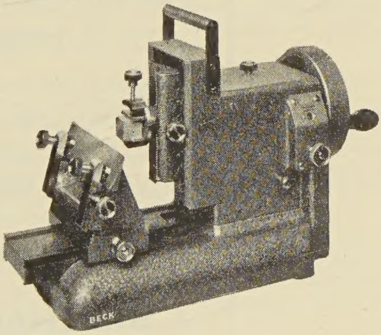

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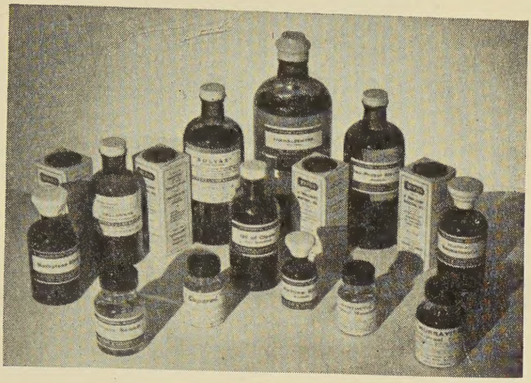

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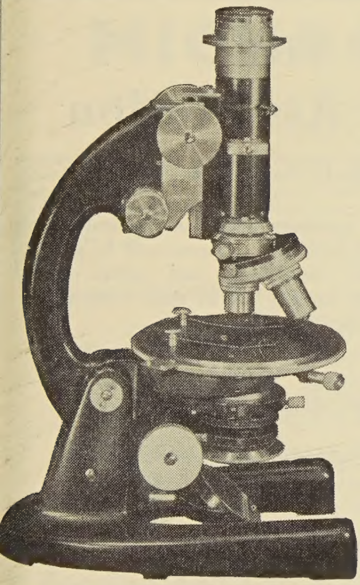

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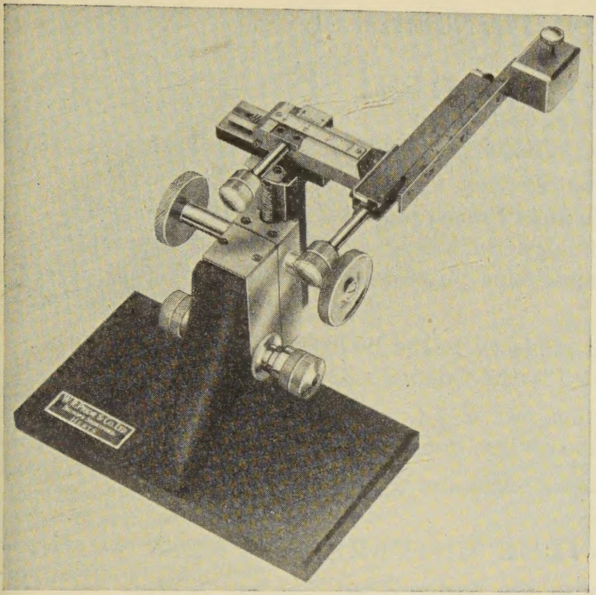

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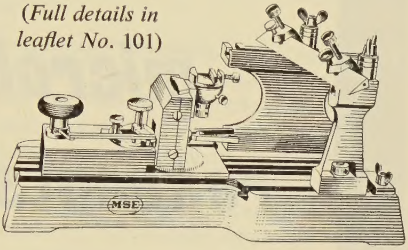

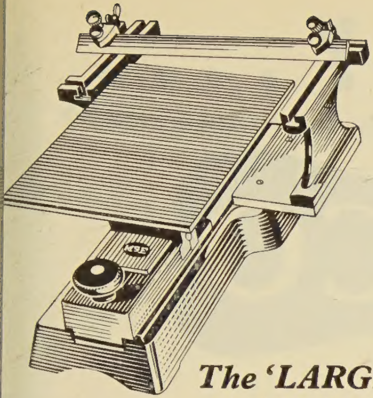

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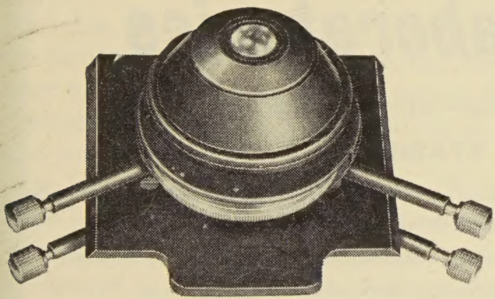

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Observations upon the Nervous Systems of Pelagic Tunicates

By QUENTIN BONE

(*From the Department of Zoology, University Museum, Oxford*)

With three plates (figs. 1, 3, and 6)

SUMMARY

The sensory and motor systems of the pelagic tunicates *Doliolum*, *Salpa*, and *Thalia* re described, chiefly from observations made upon the living animals by phase-ontrast microscopy. These observations confirm and extend previous work, showing at the sensory systems of these animals are anatomically highly specialized. Details f the sensory cells are shown from living specimens. It is shown that the motor ystems of *Doliolum* and the salps are essentially similar, but that of *Doliolum* is less ecialized. The condition of the visceral nervous system in these animals is discussed, nd it is concluded that the salps possess peripheral nerve-cells upon the viscera; lustrations are given of these cells from fixed specimens.

INTRODUCTION

KNOWLEDGE of the nervous systems of the various types of tunicate is due largely to the work of Fedele, who published a series of papers n this subject, describing both the structure and also something of the unction of the nervous systems in the different groups. His results have eceived less attention than they merit, perhaps because many of his papers ere without adequate illustration, but also because the refractoriness of hese animals to the usual techniques for the demonstration of nervous tissue as led to later investigators being unable to confirm his results. For this eason, this account of the peripheral nervous systems of the pelagic tunicates s illustrated in some detail, mainly from living specimens.

MATERIAL AND METHODS

Doliolum mülleri (Krohn) and *Thalia democratica* (Forsk.) were collected rom plankton hauls at different depths (from the surface to 100 m); they are ommon in the Bay of Naples, and during the time that I was able to examine he plankton (Feb.–Aug. 1955), specimens of most of the stages in the life-ycles were obtained.

The larger *Salpa africana-maxima* (Krohn) were collected from the sea urface by dipping them out individually in a bucket.

The natural transparency and thinness of these animals make them objects ell adapted to the method of phase-contrast microscopy, the smaller forms eing examined entire (under a coverslip for the one-twelfth immersion bjective), the larger *Salpa* was dissected and small pieces examined in the ame way. Under these conditions, the animals remained in a state suitable

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for observation for 30 min or longer, changes in the activity of the heart and body-muscles indicating when they were no longer in suitable condition. In addition, specimens were fixed and stained in various ways; the most useful preparations being obtained from animals fixed and stained by Fedele's method (Fedele, 1920), and from material treated by a modification of Holmes's silver method (Holmes, 1947). The modification of Holmes's technique is given in detail below, for it yields results with tunicate material that has hitherto proven refractory to silver-staining (Beccari, 1955; Millar, 1953). The results obtained are inferior to the best preparations given by the method in vertebrate material, but they are superior to other methods that I have tried upon tunicate material.

Modification of Holmes's silver method

1. Fix in sea-water Bouin. Results may be obtained after formaldehyde fixation, but they are less certain.
2. Wash out in 70% ethanol. If this wash is carried out (with several changes) at 37° C, it is complete after about 24 h.
3. Wash in running tap-water, 12 h.
4. Distilled water, many changes, 12–24 h.
5. 20% silver nitrate, 12–24 h.
6. Distilled water, many changes, 12–24 h.
7. Impregnate in the original Holmes's buffered silver-pyridine solution, made up with 2% silver nitrate (in place of the 1% of the original method), 96–120 h.
8. Reduce in Holmes's reducer (1·5 g quinol; 10 g sodium sulphite crystals; 100 ml distilled water), for 2 min. The reducer must be above 37° C.
9. Wash out reducer in running tap-water, 20 min.
10. Through grades of ethanol, to balsam through phenol-xylene from 95% ethanol.

The method as given above is suitable for *whole mounts*, which may be large (e.g. an entire *Salpa* or *Ciona*). It is usually convenient to impregnate the animal as a whole, and then later to dissect those parts to be studied when the preparation is in balsam.

The length of time in the impregnating bath is not critical, but the degree of impregnation varies, depending upon the length of time in the bath; it is thus convenient to impregnate a number of pieces at one time, removing pieces at intervals until the desired result has been obtained. Where several pieces are impregnated in the same bath, or where impregnation is carried out for longer than 24 h, the solution must be replaced at 24 h intervals. The impregnating solution may be made up in large quantity, and will keep for several weeks at least. Gold-toning and subsequent oxalic treatment may be carried out after step 9; but for whole mounts this is usually not an advantage as the tissues become too darkly stained to allow views of underlying layers.

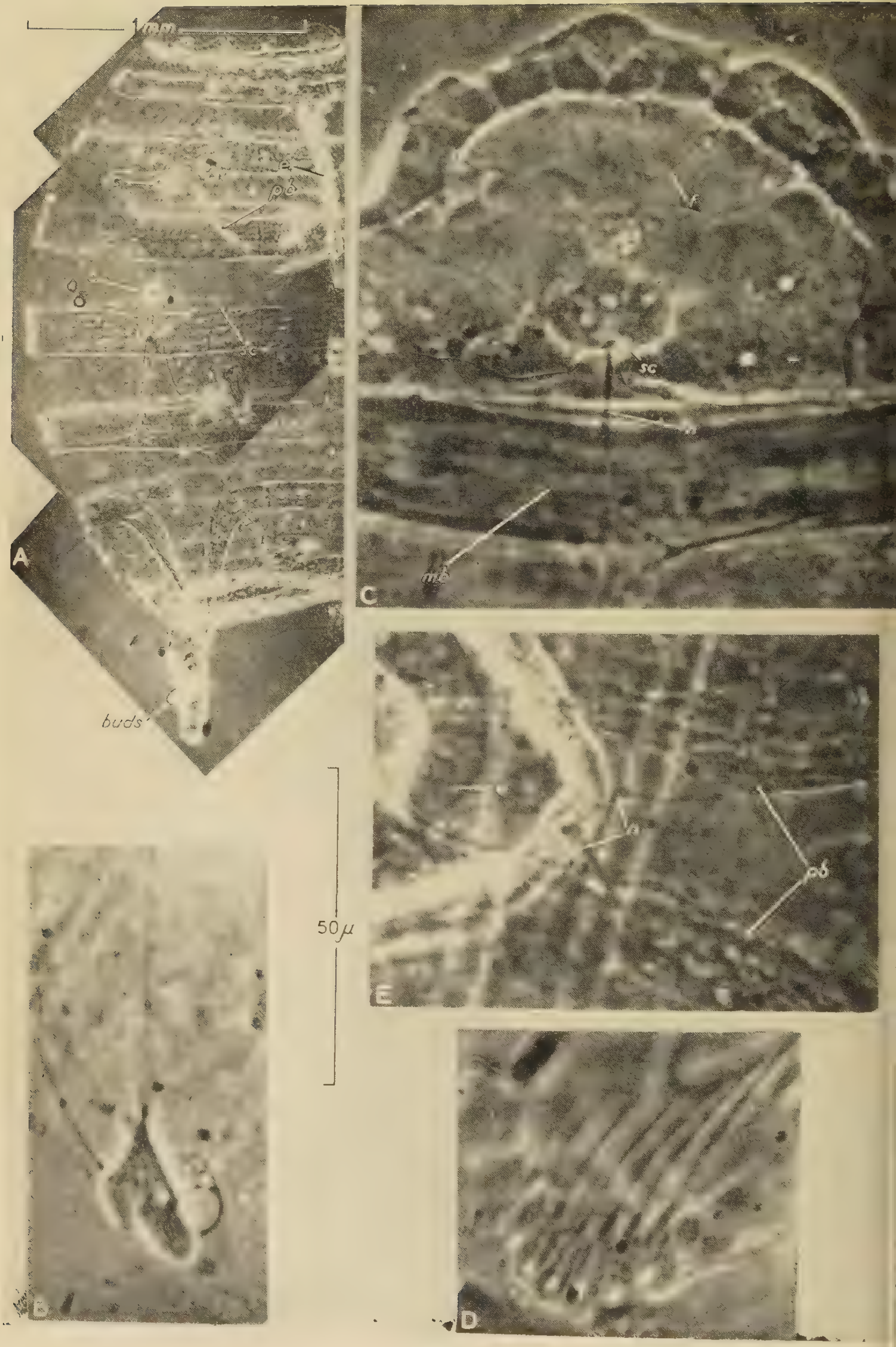

Fig. 1

Q. BONE

OBSERVATIONS

oliolum

The nervous system in all stages consists of the dorsal spherical brain, ing between muscle-bands 4 and 5, which gives off mixed paired and unaired nerves to the body-muscles, sensory cells, and viscera.

The general arrangement in the living animal is seen in fig. 1, A.

The sensory system. All the different stages in the life-cycle possess a rather omplex sensory system, in which there are sense cells of several different natomical types. In the oozooids and gonozooids the pattern of these sense ells is similar (as it was in the single phorozooid secured). In the gastrozooid, ccording to the figure given by Grobben (1892), the pattern is somewhat ifferent. The late oozooids show the same degeneration of the sense cells as shown by the internal organs; this stage is specialized for the transport of e next stages upon the long cadophore stalk, and consists, when mature, of e body-wall and greatly enlarged muscle-bands; all the internal organs isappear. Apart from the specialized otocyst of the gonozooid and the adophore organ of the oozooid, which are not represented in the other stages, ere are three types of sensory cell found in both these stages.

The first, the surface sense cells, are found at definite sites on the body, ing in groups of 1–4 cells together. In the living animal these cells have a ery characteristic structure (rather dissimilar to the earlier figures from tained preparations given by Uljanin (1884)), seen in fig. 1, B, and fig. 2, A. t is interesting that this structure is found also in salps, and in *Ciona* (Millar); appears to be characteristic of the Tunicata as a whole.

The spike of the cell proceeds to the surface of the test from a dense granule a vacuole adjacent to the nucleus. As it passes through the neck of the cell, lies in a less dense tube, leaving by a denser cap at the top of the cell. Each ense cell (which is of the 'invertebrate' type, sending its own axon to the rain) is accompanied by a supporting cell. The supporting cell is similar to e sense cell but possesses no axon, and only a few short processes in place of e spike: it stains more darkly with haematoxylin. In *Ciona* the supporting ells are more similar to the polygonal epithelial cells than to the sense cells. t is noteworthy that supporting cells are not found in association with the ense cells of salps.

FIG. 1 (plate). A, low power view of oozooid, showing brain and nerves radiating from it; adophore stalk and bud; positions of surface sense cells.

B, surface sense cell and supporting cell. The sense cell structure is not clear in this view.

C, front lips of unhatched oozooid. Triad of sense cells in the middle of the lips (their spikes assing downward out of the focal plane); nerve-fibres in connexion with the cells edging the ps.

D, cell group from the cadophore organ. The characteristic structure of the sense cells is ident.

E, nerve-fibres entering the front of the endostyle after running along the peripharyngeal ands.

b, brain; *e*, endostyle; *f*, fibres to lip cells; *mb*, first muscle band; *n*, nerves; *pb*, peripharyneal band; *sc*, sense cells (a triad in C).

(All from living specimens; phase-contrast. The 50-μ scale applies to B–E.)

It is likely that the surface sense cells are sensitive to the deformation of the test produced by light touch; salps are much less sensitive to touch than *Doliolum*, which may probably be related to the much greater relative thickness of the test in the former group.

Of the other types of sense cell found upon the oozooid and gonozooid, two are similar in form to the surface sense cells. One type is found at the bases of the rear lips, sending its process to the edge of the lip (supported at the base in cone-shaped thickenings of the test of the lips). Each cell lies between two supporting cells; the arrangement around the lips is regular, so that there are 10–15 regularly spaced groups of these cells around the rear lips. Between the sense cells of this type around the lips there are other cells bearing shorter spikes, and unaccompanied by supporting cells.

On the front lips the arrangement is different, for there are no supporting cells associated with the triads of sense cells that lie close to the first muscle-band in the middle of each flap of the lip. The spikes of these cells point inward and slightly backward (toward the atrial cavity) when the flaps of the lips protrude forward.

Possibly these cells are stimulated by the deformation of their processes when water flows through the lips as the animal swims. They may be concerned with the reflex regulation of swimming movements (the animal swims forward or back by closing front or rear lips when the body-muscles are contracted).

From the base of the triad of sense cells of the front lips fine nerve-fibres run forward to the edges of the lips. The fibres branch as they cross the flaps of the lips, and a branch is in connexion with each of the cells edging the lips. The arrangement is seen in fig. 1, c.

The cells at the edge of the lips each bear short, stout processes, which are in constant irregular movement. The movements of adjacent processes are not synchronous. If these cells are to be regarded as primary sense cells (and the fibres with them as their own axons), then they may perhaps be analogous in function to the olfactory receptor cells of the vertebrate olfactory mucosa, where Pomerat (1957) has shown the cell processes to be in constant movement. Le Gros Clark (1957) has pointed out that the movements of the cell processes of the olfactory mucosa may well be related directly to their function of receiving stimuli from very low concentrations of odorous particles; it is possible that the chemoreceptors that *Doliolum* is known to possess (from experiments with dissolved chemicals) are to be identified with the cells edging the front lips, and that they may function in a manner analogous to the olfactory receptors of vertebrates.

It is not certain, however, that the nerve-fibres leading to these cells do not pass between them, and end freely; I am inclined to believe that this is less likely than that they are the axons of the cells edging the lips.

Lastly, there are two sensory arrangements, the otocyst and cadophore organ, that are found only in single stages of the life-history. The otocyst is too thick for useful phase-contrast observation, and stained preparations ave confirmed Uljanin's earlier account. The cadophore organ is found on he base of the cadophore stalk, and can be conveniently studied with phase-ontrast. It was long ago described by Uljanin, and later discussed by Fedele 1923), who reserved its detailed description for a later paper that never ppeared.

The organ consists of groups of 5–10 sense cells lying closely packed apparently without accompanying supporting cells); there are in the whole rgan, 4–7 of these cell groups. The spikes of the cells point posteriorly down he stalk, toward the groups of buds lying farther out on the cadophore. ig. 1, D shows the structure of one of these cell groups, where the typical tructure of the tunicate surface sense cell can be seen. The variations in rrangement of the organ in different animals, which are considerable, do not ppear to be related to the age of the oozooid; the organ is present before the ozooid hatches, and is retained even in the old nurses when the surface sense ells appear to have degenerated.

Nothing is known of the function of the cadophore organ; it is reasonable o suppose that the sense cells are stimulated by movement of water past their rocesses (as is produced when the animal moves forward), but how the nimal may utilize this information is uncertain.

The motor system, the arrangement of the brain, and the innervation of the nternal organs. The nerves which pass out from the brain contain fibres from he various sensory cells described above, motor-fibres to the circular muscles f the body, and fibres of uncertain nature in connexion with the viscera. ach of the circular muscle-bands is supplied by fibres which may run in everal different nerve-trunks; i.e. a specific muscle-band is always supplied y the same nerve-trunks in different individuals, but more than one nerve-runk contains fibres to the same muscle-band. It is probable that the motor-ibres are the axons of the large nerve-cells lying at the periphery of the brain as Fedele showed elegantly in the larger *Salpa* (1933)). The motor-fibres end n a characteristic way upon the muscle-bands; unlike salps where there are pecial end-formations, in *Doliolum* the motor-fibres end without special end-hickenings, and lie at right angles to the muscle-fibres (the nerve-trunk runs arallel with the muscle-fibres of the muscle-band, and gives off fine lateral nd-fibres). The arrangement is seen in fig. 2, B. The system is simpler, and n a smaller scale than in the salps, consideration of the problems of the umbers of end-formations upon single muscle-bands, and the overlap beween several motor-cells upon the same muscle-fibre will be deferred until he system in the salps has been described.

The internal organs are supplied by three nerves. An unpaired median erve runs backward from the brain, passes inward at the point where the ill bars are attached to the roof of the pharynx, and then presumably runs o the viscera. I have not been able to follow the further course of this nerve; resumably (by analogy with salps), it is in connexion with a system of visceral erve-cells. Fedele (1923) has given an account of this system. He has also emonstrated that the cilia of the gill bars are under nervous control, but his experimental results do not suggest that this control is mediated through the unpaired nerve. Anteriorly, two smaller nerves run forward from the brain on each side of the tube of the sub-neural gland; sending fibres to the ciliary organ, they pass on in close association with the peripharyngeal bands. They run round the pharynx to the front of the endostyle, where they can no longer be followed in the living animal. Fig. 1, E illustrates the nerves entering the endostyle at its junction with the peripharyngeal bands.

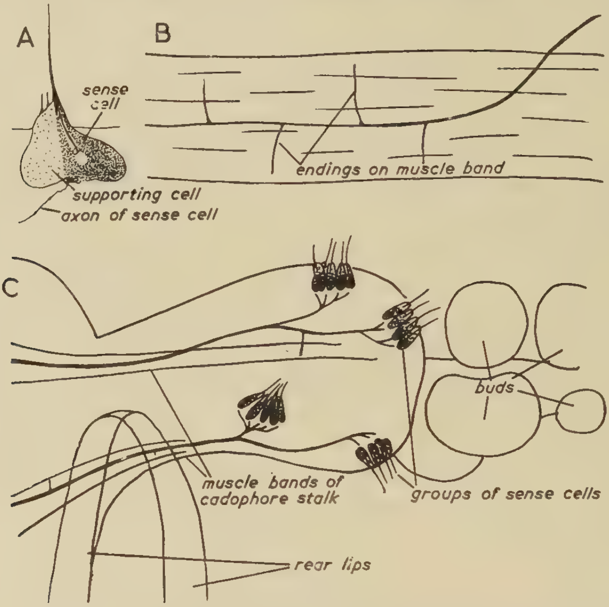

FIG. 2. A, sense cell and supporting cell from surface of body of *Doliolum*. B, arrangement of motor-endings in *Doliolum*. C, arrangement of the cadophore organ of the oozooid.

It is not certain what the function of these fibres may be. It seems likely, from the early observations of Fol (1876), confirmed by Fedele (1923), that mucous secretion by the endostyle is intermittent, and under nervous control; some of the fibres leading to the endostyle are therefore probably secretomotor in function. Others, however, almost certainly run the length of the endostyle, and connect with a visceral plexus on the gut. Little can be made out of the visceral plexus in living *Doliolum*, but Fedele's suggestion, that it is similar to that of the salps, is probably correct. Fedele carried out some interesting experiments upon the beat of the cilia of the gill bars, where he showed that the intermittent character of the beat of these cilia was retained even after the nerve running from the brain to the gill bars had been cut: presumably indicating that these cilia were controlled by visceral nerve-cells lying in a plexus upon the gill bars and gut. I have never observed nerve-cells in stained preparations of the viscera of *Doliolum*; this is not to say that they are not present.

In the living animal the brain is too thick for useful phase-contrast observation; when stained with haematoxylin, its structure appears much like that of *Salpa* (Fedele, 1933). Around the periphery there are large cells relatively few in number which surround the inner mass of smaller cells. Fedele showed in *Salpa* that the larger cells were motor to the body-muscles, and the smaller associative; very probably the same is true in *Doliolum*, where the cell number is very much smaller. In a late oozooid, there were approximately 50–70 large motor-cells, so that each muscle-band receives its nerve-supply from 8 to 9 motoneurones; considering the synchrony of contraction of the muscle-bands when the animal is swimming, the connexions of these motor-cells within the brain must be relatively complex.

Salpa africana-maxima and Thalia democratica

The nervous systems of the salps are essentially similar to those of the doliolids; from a dorsal spherical brain, mixed nerves radiate to different parts of the body. What differences exist are due to the larger size of the salps and to the formation of associations of individuals in this sub-class. The observations recorded below were made chiefly upon the smaller *Thalia*, which was very abundant in the plankton and, except where noted, refer to that species alone.

The sensory system. As in *Doliolum*, the sensory system is remarkably developed, and there are several anatomically different types of sense cell. On the front lips there are two types. The first lies at the anterior edge of the lips, and bears a long process extending forward in the plane of the lip. The lips bend back and forth as the swimming muscles of the body contract (there is a special system of small muscle-bands around the lips, controlling their position). The animal moves forward or back by controlling the closure of the valves of the lips. Deformations of sense cells of this type (as in *Doliolum*) are probably involved in reflex patterns superposed upon the normal forward swimming.

Secondly, there are large numbers of irregularly arranged cells all around the lips, lying between the edges of the lips and the first muscle-band; they do not bear long processes. Fedele described such cells in *Salpa*, but did not observe them in *Thalia*. He regarded these cells as chemoreceptors. They are seen in fig. 3, A.

The front lips of the larger *Salpa* contain both these cell types, and perhaps also free nerve-endings. The rear lips contain the first type of cell but not the second. As in *Doliolum*, the cell processes are supported by elongate V-shaped thickenings of the test (which stain deeply with methylene blue).

The surface sense cells are similar to those of *Doliolum* (apart from the absence of supporting cells), bearing very long spikes which reach to the test surface. The spike may arise from the top of the cell, or the cell itself may be flattened and the spike arise from its edge (fig. 3, B, C). There is not the same regularity either in number or distribution of the surface sense cells as is seen in *Doliolum* (perhaps reflecting the larger size of the salps), but the cells are normally concentrated at the front end of the animal, above the ciliary organ dorsally, and dorsally at the posterior end of the animal. In addition to these sense cells which are in a general way comparable to those found in *Doliolum*, there are several special sense organs which are of a greater degree of anatomical specialization than any of that animal. Firstly, there are the 'eyes' which lie above the brain. They have been described in some detail by Metcalf and Lentz-Johnston (1905). No satisfactory view has been advanced upon the function of these organs. Then there are several sensory arrangements which are characteristic of the aggregate forms and which probably play a part in the functional interrelation between the associated individuals. Bolles Lee long ago gave an excellent description of the most striking of these, the tentacular organ, which consists of a long lateral projection from the body, at the tip of which a group of sense cells send their processes far out in a 'flag' of test material (Bolles Lee, 1891). Similar organs are present in *Salpa*, but their shape is different, for the organ resembles a shallow dome, in which the sense cells lie; a 'flag' of test material projects from the top of the dome. No doubt in the larger animal the long process seen in *Thalia* would be too fragile, and this accounts for the difference in form. These organs are paired and lie laterally, somewhat asymmetrically, at the front of the animal.

There have been various suggestions upon their function. It seems most probable that they respond to water-flow past them (i.e. to the bending of the flag of test material), and that stimulation of these organs on one side of the body of a zooid of the chain by the water currents produced by the swimming contractions of adjacent zooids, results in the maintenance of swimming movements by the chain as a whole. However, there is little experimental evidence to support this view. Various experiments were performed upon zooids isolated from a chain of individuals (e.g. unilateral extirpation, unilateral stimulation by a water-jet, &c.), but these did not yield clear-cut results. It is very probable that these organs are easily damaged when the animal is placed in a container after it is collected from the surface of the sea, and that this accounts for the variable results obtained in such experiments.

The individual zooids are joined at a number of points (two in *Salpa*, four in *Thalia*); at these junctions there are special sensory arrangements. Fedele showed that in *Salpa* there were collections of sense cells at these junction-

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FIG. 3 (plate). A, sense cells from front lips (many of the cells in the field are not in the focal plane). The muscle-band is part of the system controlling the shape of the mouth, which lies to the left.

B and C, surface sense cells from anterior end of animal. Note absence of supporting cells, and in C, flattened form of the cell (which is viewed from the side).

D, attachment of zooids of *Thalia*, showing nerve-fibres running up to the junction point.

E, end-formation upon muscle-band. This is one of the small muscle-bands at the anterior end of the body. There are 6 muscle-fibres, all innervated by this one axon.

F, visceral nerve running along gill bar of *Salpa*. Note large sheath swelling; the smaller sheath-cell nuclei are not visible.

ap, attachment point; *mb*, muscle-band; *n*, nerves to brain; *nf*, nerve-fibres; *sc*, sense cells.

(All except F from living specimens; phase-contrast. F is from a preparation fixed with osmic-acetic. The 50-μ scale applies to A–E.)

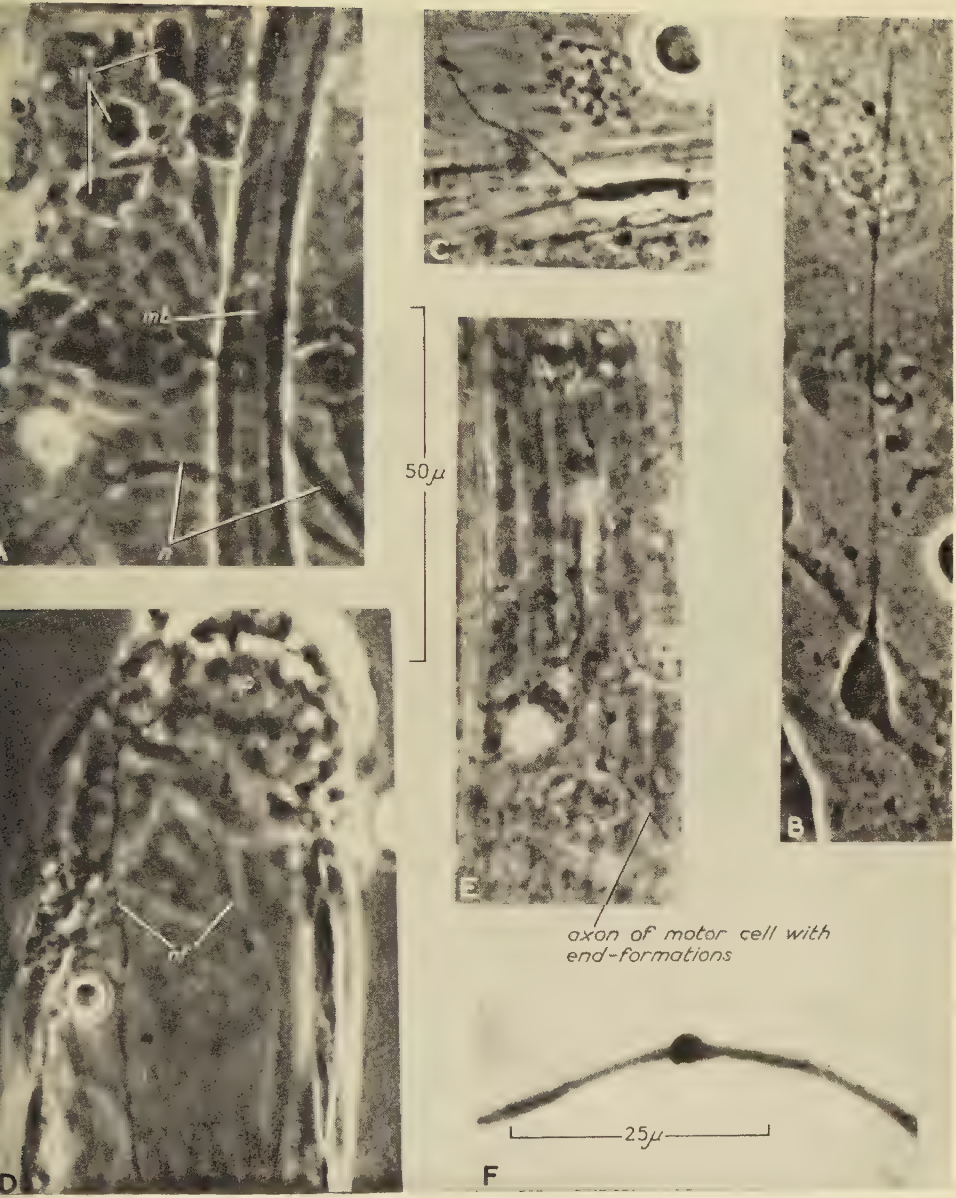

FIG. 3

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oints between the two zooids; when the animals are separated, the sense ells remain below the scars on the test. In preparations made by Fedele's nethod, sense cells are seen not only in the large aggregations that he described, ut also scattered at irregular intervals along the length of the junction.

When the zooids are joined in the chain it is evident that these cells must e stimulated by the strains that the junction-points receive when the ttached zooids contract during swimming, and so provide information for egulating the activities of the chain.

In *Thalia* there do not seem to be any sense cells of this type at the attachnent points (which are long and tubular, not flattened). The nerve-fibres unning into the attachments (fig. 3, D) may end freely at the junction-points.

The functions of the various sense organs of the salps can be inferred in a eneral way from the anatomical arrangements of the sense cells; but the part hat these play in the normal life of the animal is not at all clear. Fedele (1923*b*) as discussed the special sense organs found in the aggregate forms of salps, nd has provided some evidence for their functions. Nevertheless, little is ertainly known of the way in which these animals utilize the responses of heir sense organs.

The motor system. The axons of the large peripheral cells in the brain erminate upon the muscle-bands in definite end-formations, not unlike the motor end-plates of vertebrates. These were described by Fedele (1925), who ave an excellent account of their structure as observed after his osmiccetic method. One of these end-formations in the living animal is illustrated n fig. 3, E; as the muscles are in constant movement, it is difficult to achieve atisfactory pictures of the structure of these end-formations in the living nimal. It is clear, however, that the structures described by Fedele are not rtifact: it is easy to observe them with phase-contrast. The arrangement of hese end-formations raises questions of some interest.

Fig. 4 shows the end-formations seen on a muscle-band of a small specimen f *Thalia*. Each muscle-band is supplied by fibres which may run in several ifferent nerves, i.e. a single band is supplied by axons running in several ifferent nerve-trunks. A single axon may form end-formations upon one nuscle-band, and then pass on to give rise to other end-formations on adjacent ands. Such intercalary end-formations have also been reported from lamreys (Johnston, 1908). Observation of many specimens of *Thalia*, from which sketch diagrams were made of the distribution of the end-formations, hows that single muscle-fibres may receive their innervation from different xons in addition to different muscle-fibres being innervated by a single axon.

It seems probable that the excitatory system is adapted to produce synhronous contractions of the body-muscles from a structural basis of many notor-cells acting in unison; the system is economical, for one motor-cell may ffect several muscle-bands (each containing 12 or more muscle-fibres). The umber of end-formations on a single muscle-fibre suggests that excitation nay be rather of the localized-site type, than that the end-formations give rise o propagated spikes along the muscle-fibres.

The visceral nervous system. The condition of the nervous system upon the viscera in tunicates is not certainly known. Fedele's claim that in the pelagic tunicates and also in the *Ascidiacea* there is a visceral plexus containing peripheral nerve-cells, has not been substantiated by later workers; various workers (e.g. Millar, 1953) have denied the presence of nerve-cells in the visceral plexus. Fedele himself gave figures of these cells only in the salps (stating that similar conditions were to be found in *Doliolum* and *Ciona*). A simplified version of his figure of the arrangement of the visceral plexus in the salp (Fedele, 1933) is reproduced in fig. 5. He said little about the form and interconnexion of these cells, but later (Fedele, 1938) stated that they were equivalent in nature to the cells of the vertebrate visceral plexuses (although, as he had stated that the visceral nerve contains afferent fibres, he presumably supposed some of the cells to be sensory).

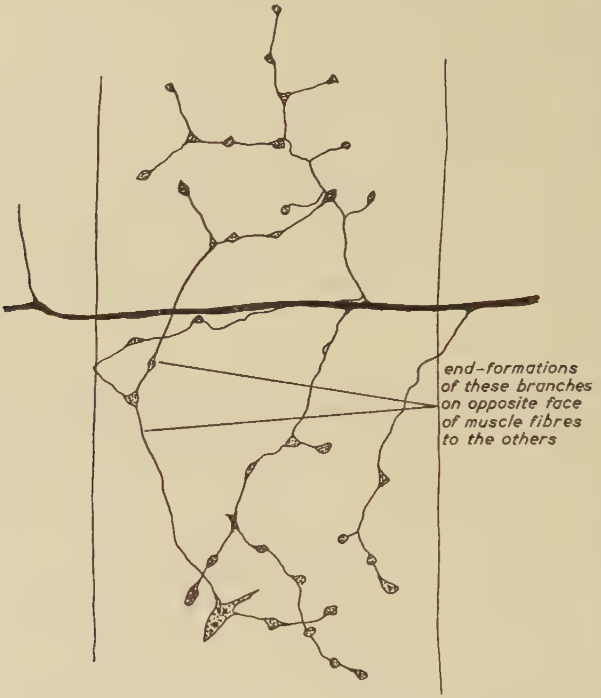

FIG. 4. End-formations upon part of a muscle-band of *Thalia*.

Fedele had claimed that the tunicate visceral nervous system was a suitable starting-point for the evolution of the vertebrate enteric systems of nerve-cells, and that it was, in fact, very similar in arrangement to the vertebrate systems (Fedele, 1938).

In my opinion, this claim is unjustified. At present we know very little about the visceral nervous systems of the tunicates. The evidence which Fedele produced to support his claim was meagre, and has not been confirmed by later work. What little is known will be discussed in this section; it is insufficient to provide a basis for any comparison with the vertebrate visceral nervous systems.

It is a simple matter in almost any type of preparation, whether fixed and stained by Fedele's method; observed in the living state with phase contrast; or stained with silver, to observe that a large nerve-bundle runs to the viscera along the base of the gill bar.

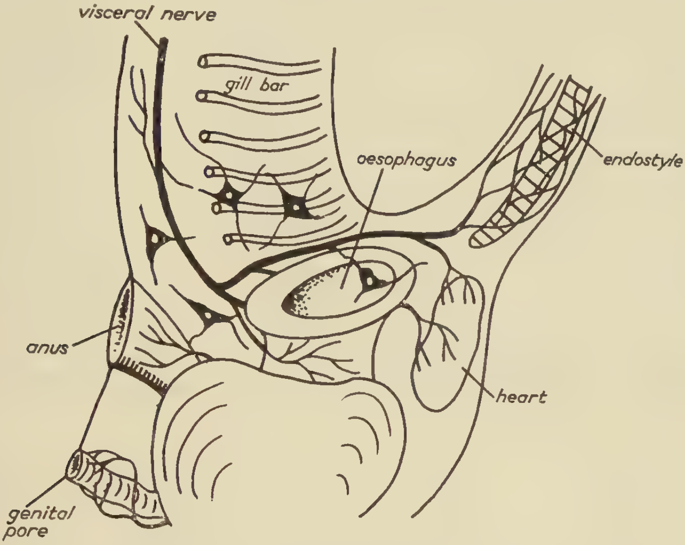

FIG. 5. The visceral nerve-plexus in salps. Slightly simplified after Fedele.

This visceral nerve-bundle probably contains (in *Salpa*) about 20–50 nerve-fibres (fig. 3, F). Similarly, by means of phase-contrast, it is simple to observe that nerve-fibres enter the front of the endostyle from the nerves lying under the peripharyngeal bands. The similar arrangement in *Doliolum* is seen in fig. 1, E. Some of these fibres are likely to be secreto-motor to the endostyle itself; others connect with the visceral plexus at the rear of the endostyle.

The ending of these fibres in the viscera is not easy to discover. Many of them can be seen in silver and osmium preparations, coursing over the gut; when their endings can be seen (rarely), it appears that they end freely, in relation to the underlying gut epithelium. The plexus is more sparse than that seen in *Ciona* by the same techniques. A part of the plexus on the gut-wall is seen in fig. 6, A.

Fedele's figures of the nerve-cells in the visceral plexus of salps are puzzling. I have never observed any cell type corresponding to the large multipolar cells which he regarded as nerve-cells.

There are several cell types which resemble nerve-cells on the gut. One of these (not similar to that described by Fedele) is regarded as actually nervous in nature. As is well known, there are in the tunicates many cell types (connective tissue and wandering cells) which in well-fixed or living tissue bear processes, exhibit different degrees of osmiophilia, and to a greater or lesser extent simulate nerve-cells. This problem is briefly discussed in a consideration of the innervation of the tunicate heart (Bone and Whitear, 1959). It is therefore necessary to consider the criteria that may be adopted to distinguish neurones from these different types of cells.

The most satisfactory criterion (upon which I have not been able to satisfy myself in the case of any of these types of cell) is that processes of the cells should be observed to run to and form nerve-bundles which can then be traced to the brain; or that nerve-fibres in these trunks can be observed to synapse with these cells. Other criteria, such as the length of the cell processes and their regularity of diameter, or the interconnexion of these processes with one another in some type of synaptic arrangement, are less satisfactory, as is also the degree to which the various types impregnate with silver solutions.

Nevertheless, the use of these and similar criteria, combined with comparison of cell types in the various tunicate groups, does give reasonably definite evidence of the presence of peripheral nerve-cells in the salp visceral plexus. Of the three types of cell that bear processes which are found on the viscera, the first is clearly connective (fig. 6, B). These cells bear short processes which do not join in synaptic relations with other cells of the same type. They stain weakly with silver and osmium tetroxide. They are found all over the viscera. Very similar cells are seen upon the pericardium of *Ciona* (Bone and Whitear, 1959). The second cell type much resembles the vertebrate fibroblast, possessing as a rule two thick, irregular processes which end freely. These cells are smaller than the first type, they stain lightly with osmium, more heavily with silver. Often groups of these cells can be found with their processes oriented in the same direction. They are here regarded as wandering fibroblast-like cells, the orientation of the processes being the result of mechanical forces.

Lastly, there are cells which are somewhat similar to the second type, but which possess extremely long processes (sometimes branching), which appear to be related to other processes of similar cells. These processes may be followed in whole mounts for distances of 200 μ or more from the cell-body before they are lost. They are of regular diameter, occasionally beaded, and end sometimes apparently freely, at other times in relation to other cells and their processes. It is this cell type which is regarded as nervous in nature;

FIG. 6 (plate). A, a part of the visceral nerve-plexus of *Salpa*.

B, connective tissue-cells upon the viscera.

C, composite field of different focal planes of the visceral nerve-cells and their processes. *nc*, nerve-cells.

D, visceral nerve-cell from base of gill bar. The main process of the cell (passing to the top of the gill bar) is in focus at the left.

E, striated muscle of the heart of *Salpa*, showing fibre system upon the muscle-fibres.

(All from fixed preparations of *Salpa*; A, D, and E, whole mounts reduced silver method; B and C from osmic-acetic whole mounts. The longer scale applies to B–E.)

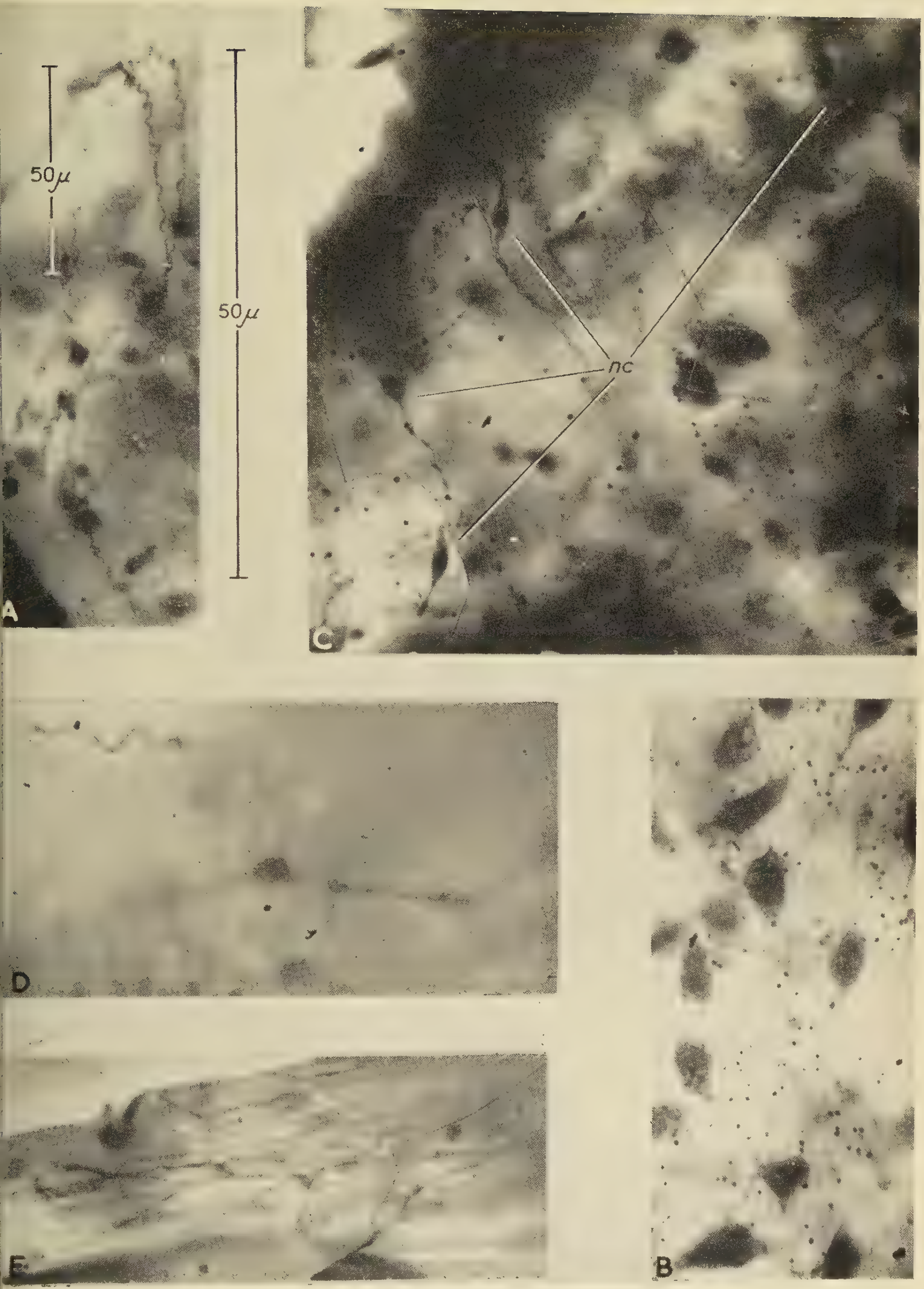

Fig. 6

Q. BONE

several of these peripheral neurones are seen in figs. 6, C, D. They are found at the sites where Fedele illustrated his nerve-cells, but are unlike his descriptions and much less common than he stated. In *Thalia* there are several cells of this type at the base of the gill bar, where it joins the visceral mass. They send long processes up the gill bar to the junction of the gill bar with the roof of the pharynx. These cells are apparently usually tri-polar, sending one long branching process up the gill bar, and shorter processes to the viscera. If these are correctly regarded as nerve-cells, then they appear to be sending their axons to the central nervous system, and may thus be supposed to be sensory; not as Fedele supposed, similar to the vertebrate enteric plexus neurones. The nerve-cells upon the gut itself may be arranged in the same way, but, although I have never observed any nerve-fibres of central origin synapsing with them, this is not to say that there are no such junctions, and it may be that there is a synapse at the periphery with the axons of central cells; I incline to the view that this is not likely.

On the whole, the evidence suggests that there are peripheral nerve-cells upon the viscera of salps, as Fedele supposed, but that these cells are unlike the descriptions that he gave. It is interesting that the salps are the only tunicate group in which Fedele actually figured the nerve-cells that he described upon the viscera; in *Ciona* he never gave figures of the nerve-cells that he claimed were abundant upon the gut and gonads. In *Ciona* Millar's careful study did not reveal any nerve-cells upon the viscera, though he found an abundant nerve-plexus. I have made many preparations of *Ciona* by the silver technique described above, and, like Millar, have not been able to confirm the existence of nerve-cells upon the gut. However, there appear to be nerve-cells in the plexus upon the gonoducts. There, cells of the third type described here are seen. Their processes can be followed in many cases to the dorsal nerve (whence they presumably run to the brain). In *Ciona* these nerve-cells of the gonoduct plexus are often multipolar, stellate, with many short processes, and one or two longer processes in connexion with other cell-bodies and with the dorsal nerve. Some of the nerve-cells, however, are very similar to those of the salp plexus, figured in fig. 6, C, D.

The innervation of the heart. Fedele figured nerve-branches from the visceral nerve passing to and innervating the heart. He did not give any details of the way in which these nerve-fibres connected with the heart-tissues.

Nerve-fibres can be seen running toward the heart (though not in so definite an arrangement of nerve-branches as Fedele described). When they reach the heart, they course over the pericardium in a rather open plexus. A very similar arrangement is found in *Ciona*, where nerve-fibres were observed only upon the pericardium, and never seen upon the heart-muscle itself (Bone and Whitear, 1959). It is possible that in that case (and perhaps also in salps), these fibres may simply be a part of the general plexus covering the viscera, and not specifically related to the heart.

In *Salpa*, however, I have observed what appears to be a conducting system of nerve-fibres upon the muscle-fibres of the heart. I have not been able to show that this system is in connexion with the nerve-fibres seen upon the pericardium. A part of the system on the heart-muscle of *Salpa* is seen in fig. 6, E.

Branching fibres course about over the heart-muscle-fibres, and in some cases appear to follow spiral courses upon single muscle-fibres; at other positions, dichotomous branching results in a palmate effect.

I have never observed this system on the heart of *Ciona*, *Phallusia*, or *Thalia*; only in certain favourable preparations of *Salpa* is it impregnated, and then only in certain areas of the heart-muscle. It appears that the system is actually found over the whole area of the heart-muscle, but that it is irregular in staining. Physiologically, the conducting system of the tunicate heart is very puzzling, but such experiments as that of Ebara (1954), where excision of the middle portion of the heart in *S. fusiformis* caused both contraction waves to disappear at the operated portion, suggest that a special pathway is involved in the transmission of the contraction wave.

Several alternative explanations are clearly possible for the appearances upon the heart-muscle when it is impregnated with silver. It is suggested that the system is a special conducting system of nerve-fibres, for it is very similar to that seen upon the large muscle-cells of the appendicularian tail. In that case the system impregnates more fully, and ramifies over all the muscle-cells, clearly forming a conducting system. It is hoped to study the system in *Salpa* in more detail, when I am able to obtain more material suitably fixed. At present it is not possible to say whether the system connects with the raphe, or with the nerve-fibres running over the pericardium, or whether it is specialized at the centres of contraction.

Sheath-cells upon the nerve-bundles. Along the course of the nerve-bundles in salps there are at irregular intervals small swellings (one is seen on the visceral nerve in fig. 3, F); there are also very small dark-staining bodies along the nerve-fibres themselves. These smaller bodies are similar in size to the small varicosities seen along the nerve-fibres of *Ciona* and are probably the nuclei of the sheath-cells surrounding the nerve-fibres. The larger swellings are probably nuclei of the epineurium surrounding the nerve-bundles. If the smaller bodies are considered to be sheath-cell nuclei, it is interesting to consider the question of the relative numbers of the sheath-cells and the nerve-fibres that they surround. In vertebrates it is known that the smaller peripheral C fibres lie within folds of the Schwann cell cytoplasm, each Schwann cell enclosing several nerve-fibres (Gasser, 1952).

In the tunicates it seems that the arrangement is different; each nerve-fibre is related to a single sheath-cell, for there are many more sheath nuclei than would accord with the hypothesis that the situation was the same as it is in the vertebrates. If it is assumed that along a short course of the nerve-fibre (say $50\,\mu$) each fibre will possess but a single sheath-cell, then the fibre number within a nerve may be obtained roughly by counting the sheath nuclei. This estimate will obviously be approximate, for it is not known for what distance the sheath cytoplasm of one sheath-cell may extend along the nerve-fibre (the bres are all 'non-myelinated'; there are no nodes). It seems that the estimate f 20–50 fibres for the salp visceral nerve obtained by this means is not far om that suggested on other grounds. The exact relationship of the sheath of e nerve-fibres at the end-formations upon the muscle-bands is not clear. here are no teloglia.

I am most grateful to the Director and staff of the Stazione Zoologica for eir hospitality and assistance during the earlier part of this work, which was rried out during the tenure of the Naples Biological Scholarship, and a rant from the Department of Scientific and Industrial Research. Further istological studies were carried out at the Department of Zoology, Oxford, uring the tenure of a prize fellowship at Magdalen College.

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The Morphology of the Gut of the Brown Trout (*Salmo trutta*)

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With two plates (figs. 3 and 4)

SUMMARY

1. In the trout gut a short oesophagus containing only striated circular muscles pens into a large cardiac stomach possessing inner circular and outer longitudinal mooth muscle-coats, as well as a muscularis mucosae. About 45 pyloric caeca come f the intestine, which, while containing muscle-coats, does not possess a muscularis ucosae. In the rectum, the longitudinal muscle is as thick as the circular muscle-coat, ut in other regions the circular muscle is dominant, especially in the pyloric stomach here it is over 10 times as thick as the longitudinal layer.

2. The mucosa is distinguished by the presence of a prominent layer of dense colgen, the stratum compactum, which is perforated only by nerves and blood-vessels. his layer forms a firm and relatively inextensible (approximately 10% extensibility) asis to the gut-wall. It limits the extensibility of the smooth muscle to 75% radially the stomach and 25% radially and longitudinally in the intestine. In contrast, the omachs of the pike and perch, which do not possess a stratum compactum, extend p to 200%.

3. A detailed description of the regional junctions and sphincters gives a basis for e interpretation of events occurring in the living system. Valves at the junction of the neumatic duct with the oesophagus, and between the duodenum and pyloric stomach, rve to prevent the regurgitation of gas and semi-digested food respectively. A comlex sphincter mechanism exists at the pylorus, and to a lesser extent at the antrum. series of about five circular muscle-constrictors represents the anus.

4. It is suggested that the cells forming the stratum granulosum, a layer closely ssociated with the stratum compactum, are composed of active fibroblast cells roducing collagen.

5. The rectum contains a muscular annulo-spiral septum of unknown function hich protrudes into the lumen.

INTRODUCTION

THE main purpose of this paper is to display the structure of the trout gut with special reference to its mechanical potentialities. It forms the orphological basis of studies of the distribution of the nervous elements in e gut-wall (Burnstock, 1959), of the physiology (Burnstock, 1957, 1958*a*), nd of the pharmacology (Burnstock, 1958*b*) of the fish gut. The arrangement f the smooth and striated muscle, the relative thicknesses of the circular and ongitudinal coats in the different regions of the gut, and also the sphincter nd valve systems found at the various regional junctions, give a structural asis for movements occurring in the living system. An account of the nature

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and mechanical function of the stratum compactum is included in relation to studies of the extensibility of the gut-wall in the different regions.

Previous work on the morphology of the alimentary tract of the brown trout (*Salmo trutta* or *S. fario*) was concerned mainly with the structure of the mucosa. Cajetan (1883) found large glands at the upper end of the stomach, small glands more posteriorly and slime glands only at the pylorus. Oppel (1896) included a short description of the structure of the stomach wall of the trout in a systematic account of the anatomy of the vertebrate stomach. His diagram showed longitudinal and circular muscle-coats, but the prominent muscularis mucosae and the extremely narrow glandular portion do not agree with the sections of the stomach wall described in this paper. He was the first person to give the name 'stratum compactum' to the dense layer of tissue so characteristic of the mucosa of salmonids. More recently Weinreb and Bilstad (1955) described the histology of the digestive tract of the rainbow trout (*S*. *gairdneri irideus*). Particular attention was given to the cytochemistry of the granule cells.

The morphology of the gut of the salmon, which is closely related to the trout, has also been described. Early papers by Agassiz and Vogt (1843), Valatour (1861), Barton (1900), and Gulland (1898) included descriptions of the digestive mucosa, while Greene (1912) gave a more comprehensive morphological account of the whole gut. Detailed descriptions of the layers present in the walls of the oesophagus, stomach, intestine, and pyloric caeca were included. The stomach was said to be characterized by the extensive differentiation of the mucosa, and the intestine to possess no sub-mucosa or muscularis mucosae.

MATERIAL AND METHODS

For microscopic studies the gut was removed immediately after the fish had been killed and washed through with formaldehyde-saline (formalin (40%) 10 ml., saline (0·8%) 90 ml). In the preparation of transverse sections the gut was cut up into approximately $1\frac{1}{2}$-cm lengths, pumped up with fixative to preserve a natural cylindrical shape, and identified by a series of cotton lengths before being placed in a dish of the same fixative. For longitudinal sections, lengths of about 2 cm. were flattened between slides during fixation. After about 18 h in formaldehyde-saline, the preparations were dehydrated, cleared, embedded, and sectioned at about 6 μ. The sections were stained with Ehrlich's haematoxylin and eosin, the stratum compactum becoming a distinctive pink colour. Bouin's fixative followed by iron haematoxylin or Mallory's triple stain was used for checking only.

In a general survey of the gut musculature, a comparative study of the various thicknesses of the circular and longitudinal coats of a trout 180 mm long was made. For this purpose transverse sections of different regions were prepared by pumping up segments with fixative to a size which was approximately midway between the fully extended and fully contracted (empty) dimensions (see table 1, p. 197). By means of preparations showing sections cut in three planes at right angles to one another, a detailed examination of the mechanical arrangement of the sphincters and of the regional junctions was possible.

For examination of the form and arrangement of individual muscle-fibres, the tissue was either examined fresh, after maceration in nitric acid, or in stained sections of material fixed in various ways. In the study of the limits of extensibility of the gut, 20 trout which had been starved for about 3 weeks were killed by severing the brain from the spinal cord. The gut was removed and the 'empty', fully contracted lengths and diameters of the cardiac stomach, ileum, and rectum were recorded, together with the total length of the fish measured from the tip of the snout to the extreme tip of the tail fin. The dimensions of the fully extended gut regions were examined by tying the gut behind the pyloric sphincter and at the 'ileo-duodenal' junction, and pumping up the two regions to bursting point with a strong pipette containing 0·8% saline solution. The relaxed and extended diameters were measured with callipers.

For gross comparison of the extensibility of the gut of teleostean carnivores which do not possess a stratum compactum, the pike and perch were treated in a similar manner. Measurements were also taken of the extensibility of the stomach of the char, a salmonid fish possessing a stratum compactum, but feeding on plankton.

Stained stratum compactum has the appearance of dense collagen and since high-angle diffraction patterns are a useful and direct way of identifying collagen (Randall, 1953), this method was employed to establish the fact. The stratum compactum was isolated from the gut by carefully dissecting off the other layers in 0·8% saline solution. The sample of stratum compactum to be X-rayed was dried and held in a frame so that it was slightly stretched. The final diffraction pattern was registered as a photographic film.

A method was devised for measuring the percentage stretch of the isolated stratum compactum. Wet strips, 5 mm wide but of variable length, were suspended by a spring paper clip and stretched by means of weights until they ruptured. The difference between the initial and final lengths gave a measure of the extensibility of the tissue, providing an interesting comparison with the extensibility figures of the intact gut-wall itself.

RESULTS

General structure of the gut

Gross anatomy. Fig. 1, A shows the gross anatomy of the trout gut. A short muscular oesophagus opens into the large anterior cardiac portion of the stomach with its prominent longitudinal folds. A shorter, more compact, cylindrical pyloric limb opens through a powerful pyloric sphincter into the intestine. The intestine has been divided for the sake of convenience into 'duodenal' and 'ileal' portions, the 'duodenal-ileal' junction being defined as the point beyond which no pyloric caeca appear. There is no histological distinction between these regions. The rectum, however, is clearly defined from the intestine. It is radially larger and more darkly pigmented than the intestine, with prominent, circularly arranged blood-vessels at intervals along its length. There are corresponding histological differences. In 20 fish examined, the number of pyloric caeca varied from 36 to 57.

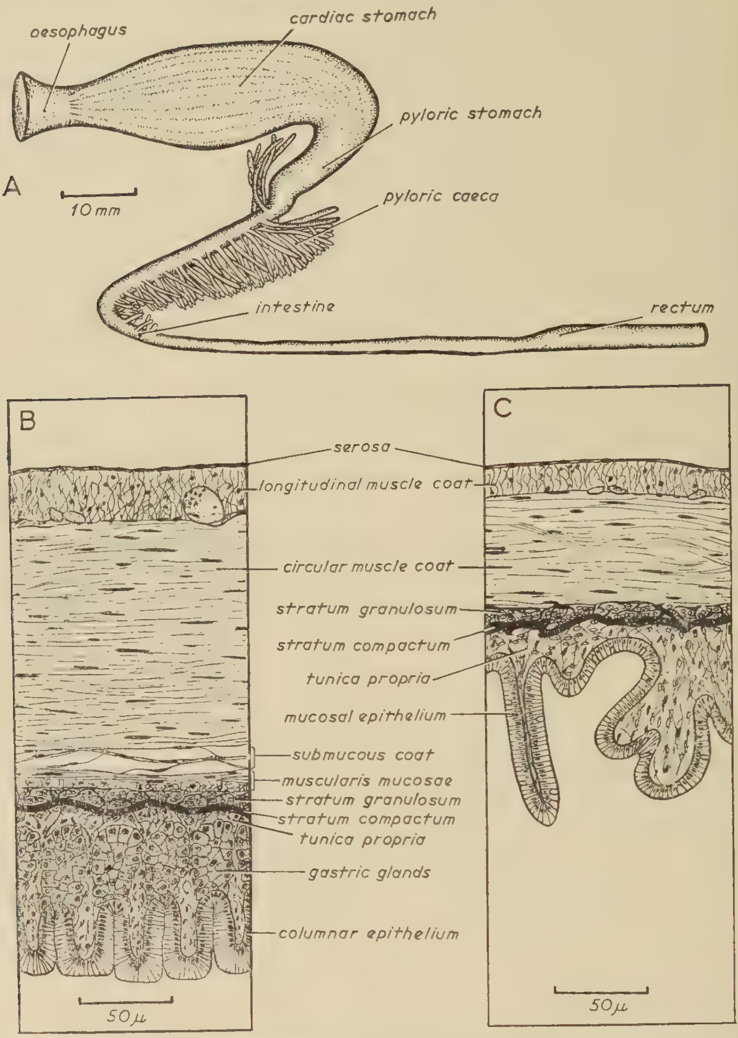

FIG. 1. A, diagram of the gross anatomy of the gut of a trout 180 mm long, showing the various regions. B, transverse section through the wall of the cardiac stomach, showing the histological relations of the various coats. C, transverse section through the wall of the intestine.

Regional histology. The *oesophagus* is short and possesses a very thick striated circular muscle-coat. There is no submucosa, muscularis mucosae stratum compactum, or stratum granulosum in the oesophagus. The columnar epithelium lies in complex folds and is formed largely of mucus-cells Dense, mainly longitudinally orientated fibres, containing few blood-vessels rm the sub-epithelial connective tissue-layer. A prominent subserous region ntaining numerous blood-vessels is characteristic of this region.

The *stomach* wall (fig. 1, B) possesses all four coats characteristically found a vertebrate gut, but is distinguished by the extensive differentiation of the ucosa. The serous coat consists of a single layer of plate-like epithelial lls. The circular muscle-coat is more than twice as thick as the longitudinal at and contains anteriorly some striated fibre-bundles extending from the sophagus. An irregular connective tissue region, containing blood-vessels d muscle-fibres, represents the submucosa and lies next to the outer layer the mucosa. This consists of a muscularis mucosae, which contains both rcular and longitudinal fibres as well as free fibre-bundles distributed irregurly in the folds of the submucosa. A prominent specialized layer of dense llagen known as the stratum compactum characterizes the mucosa. It is rforated by blood-vessels and nerves, but not by muscles. The stratum ranulosum consists of specialized granular cells, which are interposed between e more diffuse marginal fibres of the stratum compactum and are more nsely packed on the side closest to the muscularis mucosae. The tunica ropria is a thick layer of compact areolar connective tissue, swollen with any gastric glands and containing blood-vessels and nerves. Columnar ithelial cells line the lumen and lie in prominent longitudinal and circular lds.

The *intestine* differs from the stomach principally by the absence of subucosa and muscularis mucosae (fig. 1, C). A layer of serosal cells lies outside e muscle-coats, the circular layer being about twice as thick as the longidinal layer. The stratum compactum and granulosum are very prominent. he tunica propria contains no glands and is bound by a much-folded mucosal ithelium containing many mucus-cells. The 'duodenal' portion of the intesne is characterized by the presence of diverticula, the pyloric caeca, which pear to be histologically identical with the intestine.

The *rectum* possesses a powerful muscle-coat. Half-way along its length, e circular and longitudinal muscle-layers are of about equal thickness, while th coats increase in size towards the anus. The lumen is surrounded by mucosa from which annulo-spiral septa protrude. The septa extend about lf-way into the lumen, being 1·5 mm wide in a rectum of 6 mm diameter d 60 mm long (fig. 2, A, B). There are about 30 turns to the spiral, the ptum being formed largely of circular muscle-fibres surrounded by glanlar epithelium (fig. 2, B, D). Prominent circularly directed arteries and veins ing between the circular and longitudinal muscles appear to be associated ith the septa (fig. 2, C). As in the intestine, there is no submucosa, but ratum compactum and granulosum layers are prominent.

Photomicrographs of stained transverse sections taken from the different gions of the gut, extended on fixation by the method described above, are own in fig. 3. They provide a comparison of the various thicknesses of the uscle-coats of the gut of a trout 180 mm long. Circular muscle is clearly minant in most regions, especially so in the pyloric stomach where it is

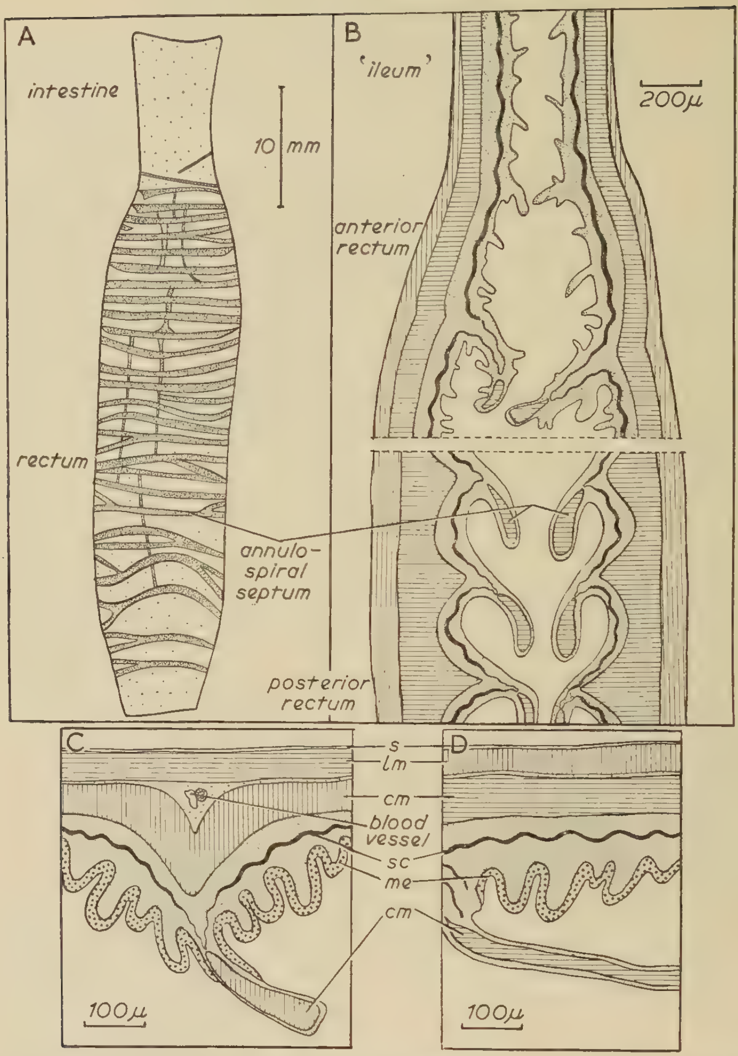

FIG. 2. A, diagram of the rectum of a trout 200 mm long, split open longitudinally and flattened, to show the gross arrangement of the annulo-spiral septum. B, longitudinal section of the 'ileo'-rectal junction and of the posterior rectum. C, longitudinal section through the rectum wall, to show the structure of the septum. *s*, Serosa; *lm*, longitudinal muscle; *cm*, circular muscle; *sc*, stratum compactum; *me*, mucosal epithelium. D, transverse section through the rectal wall.

over 10 times as thick as the longitudinal layer, measuring about $500\,\mu$. In both the cardiac stomach and intestine, however, the circular muscle-layer is only from $100\,\mu$ to $150\,\mu$ thick and represents about two-thirds of the total

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FIG. 3 (plate). Photomicrographs of stained transverse sections taken from different regions of the gut to give a comparison of the various thicknesses of the muscle-coats in a trout 180 mm long. (Haematoxylin and eosin.)

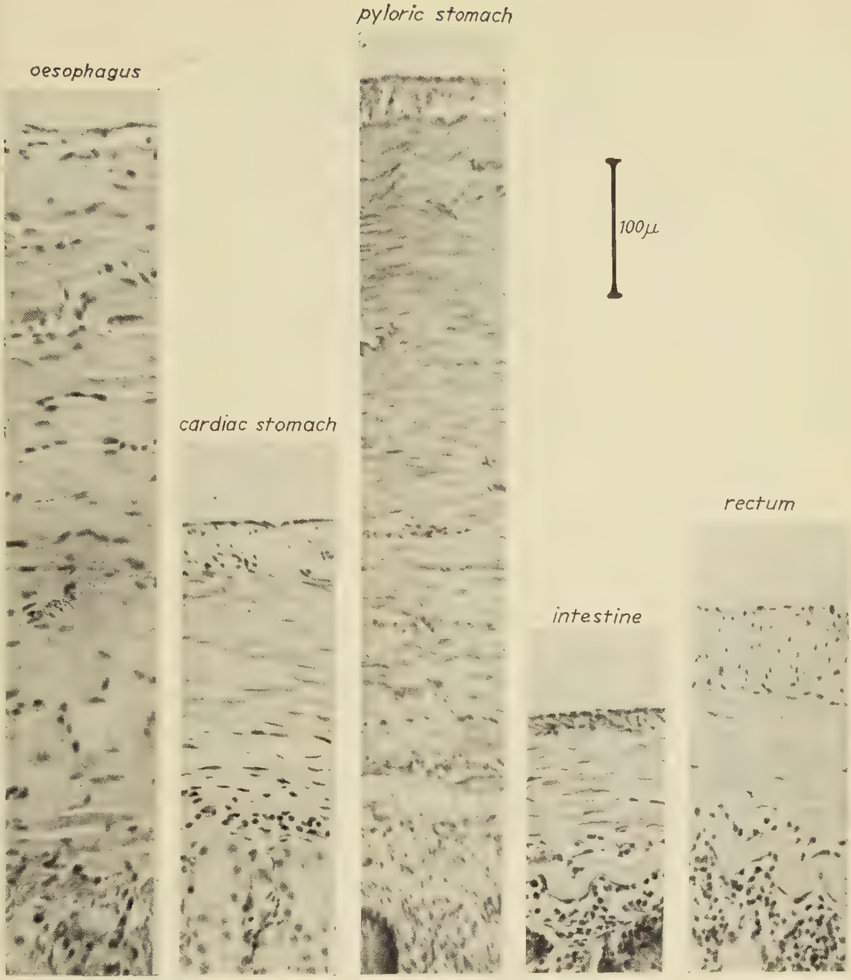

FIG. 3

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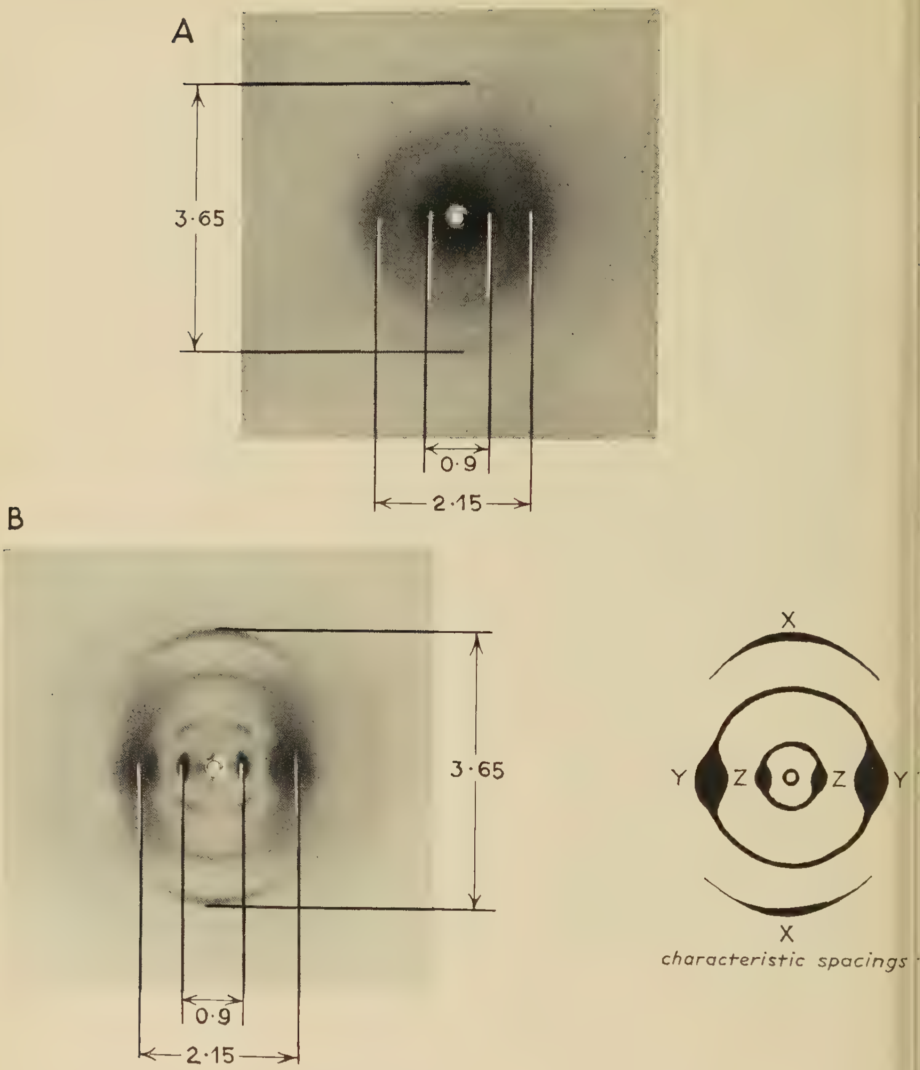

Fig. 4

G. BURNSTOCK

uscle-coat. Longitudinal muscle is absent in the oesophagus, but repre-nts about one-half of the thickness of the muscle-coat in the rectum.

Stratum compactum. The presence of a compact mucosal layer perforated by ood-vessels and nerves but not by muscles is very characteristic of sections the trout gut—indeed, of all salmonid fish. This layer is about 15 μ thick in trout 150 mm long. An X-ray photograph of the stratum compactum taken om a trout stomach is compared with a typical collagen diffraction pattern depicted by a rat-tail tendon (fig. 4).

It is clear that the stratum compactum is composed predominantly of llagen tissue. If the orientation of the fibres were completely random, the cs would form full circles, but it can be seen in the X-ray photographs that e arcs are most prominent at the poles. This means that in the trout stomach e collagen fibres are orientated largely in a longitudinal direction.

Scattered intimately among the marginal fibres of the stratum compactum e characteristic granular cells, about 8 μ in diameter, collectively known as e stratum ganulosum. Those cells farthest from the stratum compactum ntain fewer granules. The granules stain deeply with acid fuchsin and iron ematoxylin.

Form and arrangement of muscle-cells. The muscular coat of the oesophagus composed entirely of circularly arranged striated muscle-fibres. These res have a diameter of about 12 μ and oval nuclei lie beneath the sarco-mma at intervals along their length. There are alternate light and dark nds, respectively about 1 μ and 0·5 μ thick, but the intervals vary according the state of contraction of the fibre.

The smooth muscle-cells forming the longitudinal and circular muscle-ats throughout the gut are spindle-shaped, becoming very narrow towards eir pointed ends. The cell length varies from 80 to 200 μ, depending on e degree of stretch of the muscle, while the diameter of the fibre at its rgest central portion is 4 to 6 μ. The nucleus as seen in cross-section occupies uch of the middle part of the cell-body. It is elongated along the axis of the ll and in stretched muscle reaches a length of up to 30 μ. Myofibrils running ngitudinally are visible in cells examined after maceration in nitric acid, it not in fixed and stained preparations. The cells are so placed that the thick ntre of one cell is opposite the thin ends of several adjacent cells. In general, e circular muscle-fibres are more compactly arranged than those in the longi-dinal coat. Thus connective tissue forms only about 15 to 20% of the total ılk of circular muscle-coat tissue, as compared with about 25 to 30% of e longitudinal coat. These figures were obtained from measurements of the lative areas of connective and muscular tissue in sample squares taken from

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FIG. 4 (plate). A, X-ray photographs of the stratum compactum taken from a trout stomach. B, typical collagen X-ray diffraction pattern as depicted by a rat-tail tendon. The accom-nying figure indicates the *characteristic spacings.*

XX, meridional (polar) arc; YY, diffuse equatorial reflection; ZZ, equatorial side-chain acings.

different regions. Throughout the gut the muscle-fibres are bound together in small bundles by collagen. Although the fibres within each bundle lie along the same plane, the bundles themselves are often slightly, and sometimes grossly, out of line with each other. Sometimes several small bundles are enclosed in a larger, thicker collagen sheath, particularly in the region of sphincters.

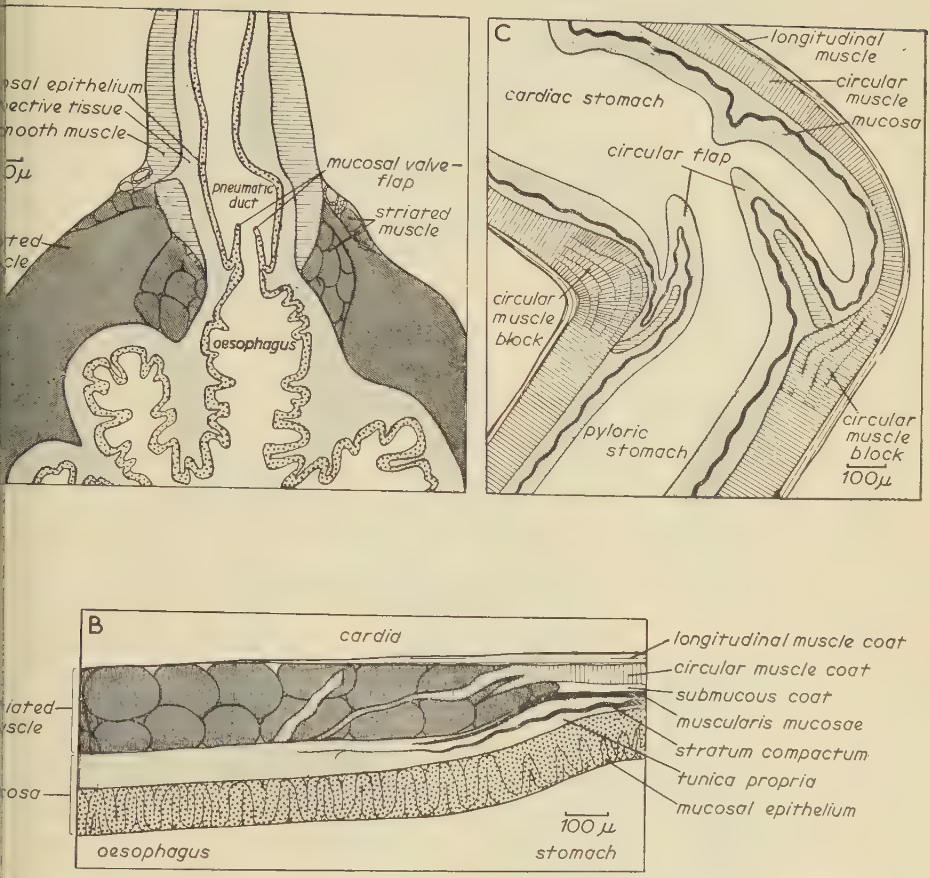
Regional junctions and sphincters.

The following parts of the gut show structural characteristics which differ from the regional histology described above.

Pneumatic duct. Salmonid fish are physostomatous. Fig. 5, A shows the junction of the pneumatic duct with the oesophagus. The diagram shows a section cut transversely through the oesophagus, so that the pneumatic duct appears in longitudinal section. The duct itself is surrounded by smooth circular muscle, while at its origin in the oesophagus, blocks of striated muscle have become reorientated to form a sphincter-like base to the duct. A non-muscular, valve-like, epithelial flap protrudes up into the lumen of the duct in this region and, together with the contraction of the basal striated muscle, presumably serves to prevent gas escaping from the swim bladder.

Cardia. The arrangement of the muscles at the junction of the oesophagus and stomach can be seen in fig. 5, B, which is a longitudinal section cut through the wall of this region. There is clearly no sphincter, but the region shows a marked morphological transition. The prominent striated circular muscle-blocks of the oesophagus give way to a narrower layer of smooth circular muscle in the stomach. The longitudinal muscle-coat makes its first appearance at the cardia together with the stratum compactum, stratum granulosum, and muscularis mucosae. The tunica propria is composed of very densely packed connective tissue. In the living system, peristaltic waves pass down the stomach from this region; no movements pass beyond the cardia in the orally directed vomiting reflex.

Antrum. Fig. 5, C shows a sagittal section of the antral region. A division into cardiac and pyloric regions of the stomach, though well marked anatomically, especially in the extended stomach, is well defined histologically. Large blocks of circular muscle lie at the apex of the inner bend of the stomach and just posterior to the curvature in the outer bend. The orientation of the muscle-fibres shows that their action is not directly antagonistic but rather that they act across each other, so that the inner block tends to pull in anteriorly to the outer one. This means that whereas the contraction of the inner block acts in a plane along the radius of curvature of the bend, the outer muscle pulls through a line at right angles to the longitudinal plane of the pyloric stomach. These morphological findings are in fact consistent with the frequent occurrence of constrictions in exactly these positions, seen in the living stomach. In fish showing the vomiting reaction, waves running orally appear to originate in this region of the stomach. In addition to the circular



IG. 5. A, diagram of a transverse section through the oesophagus at its junction with the neumatic duct. B, diagram of a longitudinal section through the cardia, showing the muscular ansition from the oesophagus to the stomach. C, diagram of a sagittal section through the antral region.

uscle-blocks, an anteriorly directed glandular flap containing circular uscles is present in this region. Contraction of this might well serve prevent large pieces of undigested food from entering the pyloric limb rematurely.

Pyloric sphincter. Fig. 6 shows sections cut in the region of the pylorus. ig. 6, A is a sagittal section, fig. 6, B a frontal section, and fig. 6, C a series of ansverse sections taken at intervals. Powerful circular muscle-blocks are ranged in an interestingly asymmetrical manner, showing a similar but more early defined mechanical system than that found in the antrum. Anteriorly ere is a ventral muscle-mass, behind which lies a very large dorsal block.

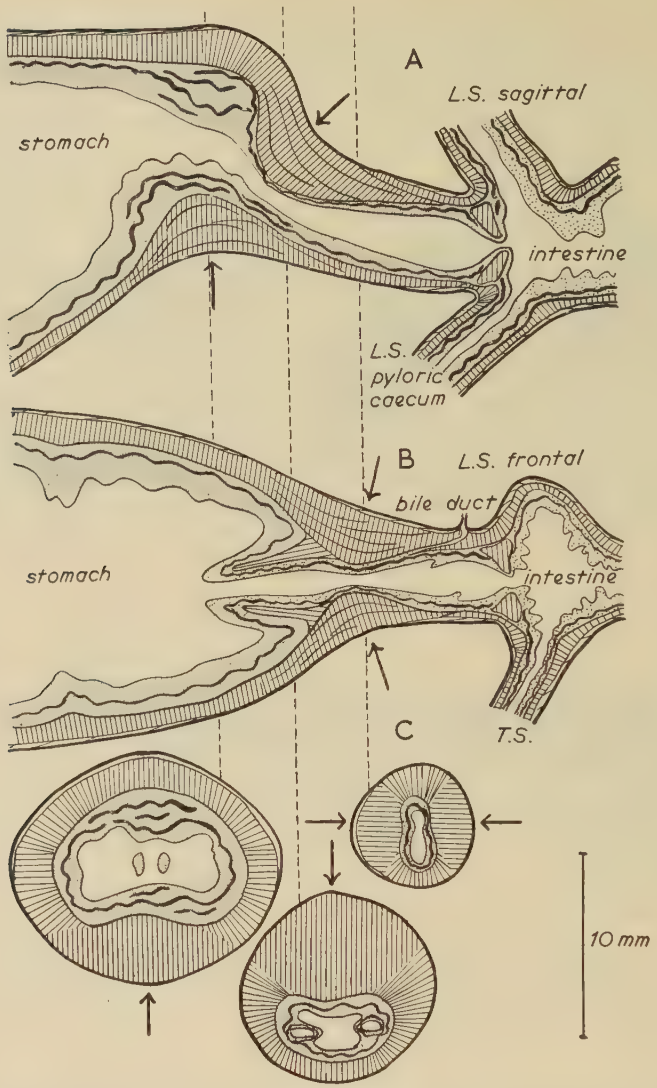


Fig. 6. Diagrams of sections cut through the region of the pyloric sphincter of a trout 254 mm long. A, sagittal section. B, frontal section. C, transverse sections through 3 regions shown at A and B.

Two lateral muscle-blocks lie posteriorly, to which are attached two forwardly directed glandular muscle-flaps. The orientation of the fibres shows that there is an interlocking action which may form a more efficient mechanism than a simple circular constriction. The ventral block contracts into the luminal space anterior to the dorsal block, which itself pulls in posteriorly. Still farther back, the lateral blocks act to complete the interlock. In the fully ntracted state the tips of the lateral flaps would probably project slightly to the lumen of the pyloric stomach. As in the antrum, the mechanical tion predicted by morphological analysis is in fact borne out by physiological servation, constrictions of this nature being characteristically seen in living eparations.

There are no longitudinal muscles in the region of the pylorus, and the rcular muscle-masses are composed of from 4 to 8 longitudinal blocks of rcular figures divided by connective-tissue sheaths. The whole region, but rticularly the area just anterior to the dorsal muscle-mass, is very heavily cked with collagen and granular cells. These presumably serve to stabilize e contractile mechanism.

At the opening of the intestine into the pylorus there is a backwardly rected circular valve flap containing circular muscle, which would be pable of preventing regurgitation of semi-digested food back into the omach.

Intestinal junctions. Figs. 6, A, B shows in longitudinal section that there is thickening of the circular muscle at the base of each pyloric caecum which presents a weak sphincter. There is no obvious physiological correlation ere.

The change from intestinal to rectal musculature is gradual (fig. 3, B). here is a general increase in the diameter of the gut, accompanied by a adual increase in the thickness of the longitudinal muscle-coat. The annulo-iral septum of the rectum begins at this junction.

Anus. The anus consists of a series of about five sphincters. Towards the sterior end of the rectum the circular muscle becomes thicker and is ranged in a series of progressively heavier circular muscle-blocks (fig. 3, . This mechanism would probably account for the fact that long cylindrical eces emerge very slowly from the anus, rather than in a sudden rush.

xtensibility of the gut

The percentage extensibilities of the lengths and diameters of the cardiac omach, 'ileum', and rectum are shown in table I (p. 197) and represent lues given by the quotient $\frac{l}{L_{\min}} \times 100$, where $l =$ the difference between the mpty' and extended dimensions and $L_{\min} =$ the 'empty 'gut dimensions.

It can be seen that the extensibility of the stomach is approximately 35% ngitudinally and 79% radially, while the intestine and rectum stretch about % both radially and longitudinally.

Measurements taken from wild Lake Windermere trout, in which the nount of food contained in the gut was variable, always fell within the limits the extensibility figures obtained in the laboratory in London.

The longitudinal stretch of isolated stratum compactum taken from the omach is about 14% along the plane of fibre orientation, but in a plane at ght angles to the fibres the stretch was only about one-half of this (table 2).

In freshly killed trout, longitudinal as well as circular folding of the stratum compactum can be seen quite clearly through the muscle-coat of the contracted stomach. Comparison of the extensibility figures of the gut itself with those of isolated collagen gives a measure of the degree of longitudinal and circular folding of the gut-wall in the various regions. Whereas the muscle-coats of the intestine and rectum and the longitudinal coat of the stomach are allowed about a 25% range of extensibility by the mechanical limitation of the stratum compactum, the circular muscles of the stomach are free to relax and contract over a 75% range.

Table 1 and table 3 show that the trout and the char, which both possess a stratum compactum, have a stomach extensibility of approximately 75%. In comparison, measurements taken from the guts of the pike and perch, which are teleosts having similar carnivorous feeding habits but lacking a stratum compactum, show an extensibility of about 200%.

Structure of the gut in developing trout

A number of trout eggs were kept in incubation trays with well-aerated running water at 12° C and examined under a binocular microscope at intervals. The gut was dissected out from a number of developing trout.

Two-and-a-half weeks after spawning, the young embryo can be seen clearly through the tough elastic membrane of the egg, beneath prominent oil globules. At this stage the gut is a very simple tube (fig. 7, A).

The gut in a 3½-week-old embryo is less easy to see, since the fish-body has become more opaque.

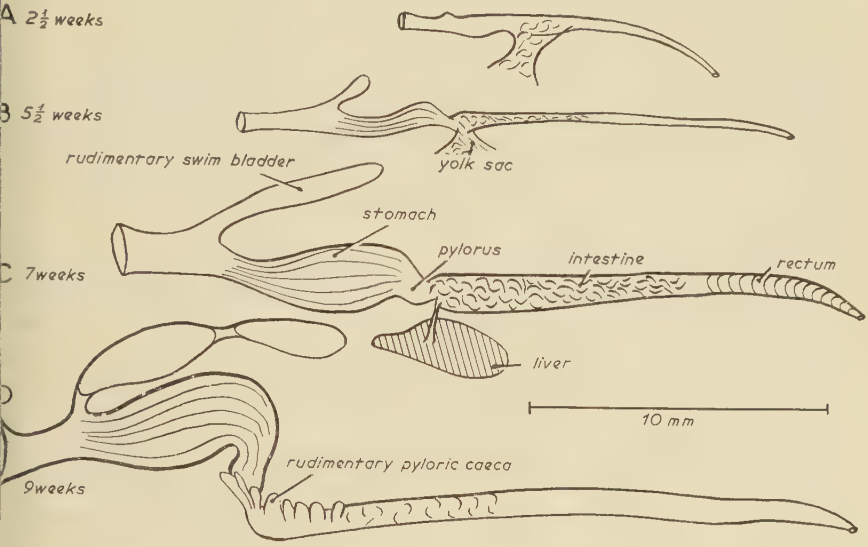
At 4½ weeks the embryo occupies most of the egg and yolk is confined to a sac, well supplied by blood-vessels. The yolk sac is connected to the gut at a point between the stomach and intestine. The form of the gut is seen much more clearly by carefully dissecting the embryo from the egg.

The young fry (or alevins) hatched out after 5½ weeks. The gut was carefully dissected out in Krebs's solution (fig. 7, B). In these fry, the oesophagus is long and a rudimentary air sac evagination is visible. The stomach shows longitudinal folds in its wall, but there is no bend yet at the antrum dividing it into cardiac and pyloric portions. The pyloric sphincter appears to be well developed, the yolk sac opening into the gut just behind it. The intestine and rectum are well developed and full of yolk. No spontaneous movements are yet visible, nor is there any reaction of any region of the gut to mechanical stimulation or to the action of acetylcholine or adrenaline.

After 7 weeks, dissection shows a general enlargement of the gut. Otherwise there is little change except that annular rings are now prominent in the rectum. The intestine is still swollen with yolk (fig. 7, C).

During the 8th week the young trout begin to feed on minute fragments of liver, and a distinct line divides the greyish stomach from the yellow intestine. No pyloric caeca have appeared, but the stomach has begun to bend just anteriorly to the pyloric sphincter.

When 9 weeks old, the fish was quite motile. The yolk sac is absorbed and e gut is used for digesting and absorbing food taken by mouth. Rudimentary loric caeca are evident and the stomach has assumed its adult form. Pinch- g the gut at this stage produces a marked reaction. A constriction is formed, llowed by contraction of the longitudinal muscle on both sides of the point stimulus, but no peristaltic wave is initiated. Acetylcholine (10^{-7} to 10^{-5} n/ml) applied to the gut clearly causes longitudinal contractions, but renaline (10^{-8} to 10^{-5} gm/ml) does not appear to affect any region of the gut. hus peristaltic waves do not appear until a very late stage in development, en after oral feeding has begun. This implies that co-ordinated neurogenic tivity in the gut does not occur until a late stage.



G. 7. Diagrams of the form of the trout gut during the early stages of its development at ° C. A, 2½ weeks after spawning. B, 5½ weeks after spawning. C, 7 weeks after spawning. D, 9 weeks after spawning.

DISCUSSION

During extensive studies of the structure of the vertebrate gut, Oppel 896) found a stratum compactum only in the gut of the falcon, cat, tench, d salmonid fish. These animals all have in common the habit of bolting ge pieces of food, some of which consist of entire and often active animals. is possible, then, that here is a case of parallel evolution of a dense collagen er as one type of adaptation to this particular feeding habit, its function ing to strengthen and preserve the entirety of the gut-wall against sudden d violent extension. Other fish with similar feeding habits, such as the pike d perch, do not possess such a limiting layer, but their guts are charac- ized by exceptionally thick muscular walls which may be an alternative aptation. From the point of view of the physiology of the trout gut, the esence of this mechanically limiting layer, which restricts extensibility to out 75%, may involve differences in the arrangement of the smooth muscles and possibly of their innervation, as compared to that of the pike and perc where the muscle is free to extend up to 200%. Studies of the orientation o the fibres in relation to the collagen lattices upon which they pull is desirabl

There has been a considerable controversy as to the function of the cell of the stratum granulosum. Greene suggested that 'their function is one o internal secretion', while Gulland (1898) described these cells as 'larg eosinophile leucocytes'. A more likely explanation is that the stratum granulosum is composed of active fibroblast cells. Fibroblast cells are almost certainly responsible for the formation of collagen fibrils and while engaged i this activity, fine granules appear in the cytoplasm (Randall, 1953). Furthe their position in the gut-wall scattered among the marginal fibres of the collagenous stratum compactum supports this hypothesis.

The pyloric sphincter is very powerful and appears to function by mean of interacting circular muscle-blocks, rather than by a single circular closur Anteriorly-directed lateral processes containing some circular muscles are als present. A reduced but similar arrangement of muscle-blocks and processe exists in the antrum. No separate extrinsic innervation of these sphincte muscles has been discovered.

A muscular annulo-spiral septum protruding into the lumen of the rectum has been described. Further study will be necessary to discover whether thi structure has a defaecatory, osmo-regulatory, or even respiratory function.

This work consists of part of a Ph.D. thesis carried out under the supervisio of Dr. M. E. Brown and Professor J. Z. Young. I would like to thank Mi North (Biophysics Unit) for taking X-ray photographs of the stratum compactum, and the Department of Scientific and Industrial Research for th grant which allowed this work to be carried out. Fig. 1, A, B has been reproduced from the *Journal of Physiology* by kind permission of the Editoria Board.

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TABLE I

Extensibility of trout-gut

Length of fish (mm)	Cardiac stomach						Ileum						Rectum					
	Length			Diameter			Length			Diameter			Length			Diameter		
	L_{min}	L_{max}	% Ext.	D_{min}	D_{max}	% Ext.	L_{min}	L_{max}	% Ext.	D_{min}	D_{max}	% Ext.	L_{min}	L_{max}	% Ext.	D_{min}	D_{max}	% Ext.
204	36	48	33	16	29	81	56	70	25	3·5	4·5	28·5	42	54	28	5	6·8	28
214	40	54	35	13	24	84·5	52	65	25	4·2	5·6	33	45	55	22	5	7·1	40
275	54	74	37	14	24	71	70	85	21	5·5	7	27	59	76	27	6·3	8	27
175	30	40	33	8·5	14	65	25	32	28	2·9	3·7	28	32	40	25	4·5	6	33
140	25	34	36	7	13	86	24	30	25	2·8	3·5	25	24	30	25	3	3·8	27
106	16·5	22	33	5	9	80	19	24	25	2·7	3·5	29	23	29	26	4·3	5·4	26
183	38	50	32	10	18·5	85	42	52	24	3·2	4	25	32	40	25	4·8	6	25
325	55	75	36	13	25	92	82	100	22	4·8	6	25	66	80	23	7·8	10	28
154	29	38	31	8·5	16·5	94	28	36	27	2·9	3·5	20	23	29	26	4·6	5·9	35
274	45	60	33	11	20	82	66	81	23	5·1	6·5	27	58	72	24	6·3	8·2	30
97	14	18·5	32	5	8	60	19	24	25	2·4	3	25	16	20	25	4·2	5·4	29
373	66	90	37	16	30	87	88	110	25	4·1	5	22	59	75	27	6·8	8·4	23
167	26	35	34·5	8	14	75	32	40	25	2·5	3	20	28	34	21	4·2	5·6	33
280	36	50	39	15	25	66	66	80	23	3·8	4·8	26	58	72	24	6·3	8	27
270	40	54	35	13	22	70	59	76	27	3·7	4·6	24	59	76	27	5·4	7·4	37
320	55	76	38	13	24	85	83	100	22	4·9	6·2	26	66	82	24	7·6	9·4	24
260	51	68	33	12	21	75	69	84	22	4·9	6·1	24	55	68	23	5·2	7·1	36
230	42	57	35	10	18	80	54	65	20	4·3	5·4	26	47	57	21	4·7	6·3	34
240	41	55	34	10·5	20	90	55	68	23	4·4	5·6	27	48	61	27	4·9	6·4	30
120	23	31	35	55	10	69	23	28	21	2·6	3·2	23	22	27	23	2·9	3·7	27
Mean			35			79			24			26			25			30

TABLE 2

Extensibility of stratum compactum

	L_{min} (*mm*)	l (*mm*)	% *Ext.*
Longitudinal stretch (along plane of fibres)	20	3	15
	30	4	13
	40	5·5	14
	50	6	12
	40	6	15
Mean			13·8
Circular stretch	40	3	7·5
	30	2	6·6
	35	2·5	7·1
	40	2	5
	20	1·5	7·5
Mean			6·7

TABLE 3

Extensibility of stomachs of three species of fish

Fish	*Length of fish* (*mm*)	*Length*			*Diameter*		
		L_{min}	L_{max}	% *Ext.*	D_{min}	D_{max}	% *Ext.*
Char	280	35	46	31	15	25	66
	300	46	58	26	11	20	82
	300	45	60	33	14	24	71
	295	40	52	30	13	22	70
	175	28	36	28	8	14	75
Mean				30			73
Perch	220	9	25	178	5	15	200
	210	12	26	117	5	15	200
	180	9	24	165	4	13	225
	180	8	22	175	4·5	13	193
	200	10	25	150	4	12	200
Mean				157			209
Pike	600	70	190	171	15	46	206
	800	100	270	170	20	65	225
	950	140	412	194	24	75	212
	350	37	110	197	8	29	250
	280	28	78	178	7	22	214
Mean				182			221

The Innervation of the Gut of the Brown Trout (*Salmo trutta*)

By G. BURNSTOCK

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With two plates (figs. 5 and 7)

SUMMARY

1. The extrinsic and intrinsic innervation of the trout gut has been examined by sing methylene blue, osmium tetroxide, and silver staining techniques.

2. Left and right vagus nerves penetrate the oesophageal wall and run between the ngitudinal and circular muscle-coats of the stomach, but do not reach the intestine. ranches of these nerves anastomose with each other and become incorporated into uerbach's plexus. Pericellular endings of vagal fibres are found about enteric neurones the stomach, while some fibres end directly on the striated muscle-cells of the sophagus.

3. A single, fine, anterior splanchnic nerve anastomoses along the major branches the coeliaco-mesenteric artery to supply both the stomach and intestine. Most of e splanchnic fibres pass directly into the smooth muscle-coats, but some fibres run to the subserous plexus.

4. Two posterior autonomic nerves provide separate innervation of the rectum.

5. Three main types of nerve-cells have been distinguished in the gut by their stribution as well as structure.

(*a*) Small uni-, bi-, and multi-polar cells (10–15 μ in diameter) form the main body Auerbach's plexus, lying between the muscle-coats. Throughout most of the gut ey are not arranged in true ganglia, since only from 1 to 3 neurones occur at the odes and many cells lie singly along the internodal connectives.

(*b*) A smaller number of large multipolar neurones (30–60 μ in diameter) lie half- nbedded in the longitudinal muscle along the whole length of the gut, their rincipal axons passing down to lie between the muscle-coats in Auerbach's plexus.

(*c*) A third cell type consisting mainly of large mono- or bi-polar cells (30–70 μ in iameter) contributes to Auerbach's plexus in the stomach only and is associated with e endings of vagus nerve-fibres.

6. Bundles of nerve-fibres pass down through the circular muscle-coat from uerbach's plexus to join a loosely arranged plexus of nerve-fibres, the submucous lexus; but unlike Meissner's plexus of mammals, which occupies a similar region, no erve-cells are present.

7. Nerve-endings, some of which may be sensory, are located at the bases of the ucous epithelial cells and are continuous with fibres forming a prominent sub- pithelial plexus from which fibres pass to both submucous and Auerbach's plexuses.

8. A subserous plexus and 'interstitial cell network' are also present in the fish gut.

9. A quantitative study of nerve-cell densities in different regions of the trout gut vealed density peaks of 93 cells per square mm and 170 cells per square mm in the sterior cardiac stomach and anterior duodenum respectively. The ratio of small large neurone types in Auerbach's plexus is about 20 to 1 in all regions. The tal number of nerve-cells in the whole gut was calculated to be about half a million.

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INTRODUCTION

A NUMBER of workers have described the autonomic system of teleost (Stannius, 1849; Chevrel, 1887; Herrick, 1898; Cole and Johnstone 1901; Müller and Liljestrand, 1918; Young, 1931; Hirt, 1934; Kirtisinghe 1940) but few of them traced out the exact course of the nerves supplyin the gut. Young (1931), however, made a detailed investigation of the extrinsi innervation of the gut of *Uranoscopus* and *Lophius*. In this work no vagu elements were found to extend beyond the stomach into the intestine, while single anterior splanchnic nerve supplied both the stomach and intestine. H failed to discover any separate innervation of the hind part of the gut an found nothing corresponding to the sacral parasympathetic outflow which i present in all tetrapods.

The intrinsic innervation of the teleostean gut was first described by Mont (1895) in the tench (*Tinca tinca*). A complicated plexus between the muscle coats was observed, with small ganglia at the nodal points, formed by multi polar nerve-cells of a single type. Fibres from the plexus were traced throug the circular muscle-coat to form a submucosal mesh of fibres, some fin branches ending in boutons on muscle-fibres of the muscularis mucosae. I addition a subepithelial plexus was described, composed of delicate nerve endings at the bases of glandular cells. Polyaxonal cells present in this regio were said to form the fibres of the plexus. The tench gut, however, cannot b taken as typical of the teleostean condition since it contains striated muscle coats throughout its entire length.

The enteric nervous system of the perch was described by Sakussef (1897) who found nerve-cells of two types in Auerbach's plexus, which correspond to Dogiel's types I and II, but only type I cells were said to be present i the intestine. Fibres were traced from the enteric plexus to the mucosa wher they ramified to form a subepithelial plexus, from which fine varicose thread came off to form networks about individual epithelial cells. Both Sakussef an Monti described nerve-endings on smooth muscle-cells.

Kirtisinghe (1940) studied the myenteric nervous system of a number o lower vertebrates. His main teleost example was *Saccobranchus* (Indian cat fish), but he included in his paper some study of *Motella* and *Ophiocephalus* For the most part he seemed content to refer back to the descriptions of Mont and Sakussef, but there were a number of additional features included in th paper. He found that the nerve-cells were usually to be seen placed singly i the meshes of the plexus, rather than aggregated at the nodal points to form ganglia. An 'interstitial cell' system lying between the muscle-coats of the gut of *Ophiocephalus* was described in his paper. The cells were reported to be o variable shape with three to four branched processes, arranged syncytially but maintaining no connexion with the enteric neurones. Those processes no uniting with neighbouring cells penetrated between the muscles. Pericellula arborizations of vagal fibres were found on nerve-cells of type II, as well as ge boutonic nerve-endings on cells of the same type, which he suggested longed to the axons of type I cells.

The object of the present paper is to examine the innervation of the gut of a ɔical teleost for comparison with those described in other vertebrates. In dition this work formed the anatomical basis of studies made on the ysiology and pharmacology of the trout gut (Burnstock, 1957, 1958 *a*, *b*).

The brown trout (*Salmo trutta*) was chosen because it belongs to a teleostean nily that retains many primitive and generalized features and because it has t adopted a specialized feeding habit. The musculature and general morphogy of the trout gut has been described previously (Burnstock, 1959).

TECHNIQUES

trinsic nerves

Tracing the paths of the vagus and splanchnic nerves was largely a matter delicate dissection under a simple lens or binocular microscope. Trout that d been freshly killed or were preserved in formalin or alcohol were used for s purpose.

Fish kept in formalin for about three months provided the best working aterial for the examination of the vagus nerves; but for the finer branches ng between the muscle-coats of the stomach wall, whole mounts of fresh ɔmach stained with methylene blue were used. These preparations were best amined with a lens while stretched about a glass tube and strongly illumited from within.

The splanchnic nerve is particularly delicate in the trout and is closely plied to blood-vessels, so that it was extremely difficult to trace by ordinary ssection methods. It was found necessary to remove lengths of carefully aned blood-vessels (and nerve) from fresh material and place them in a ution of 0·01% osmium tetroxide overnight. These preparations, after ing well washed, were mounted in glycerine or Apathy's syrup for closer amination. This technique was also used to trace out fine nerves issuing from e posterior trunk region to the rectum.

teric plexus

For a comprehensive study of the enteric plexus, both whole mounts and ial sections were required.

Whole mounts. For whole-mount preparations the methylene blue method, ɔdified from Kirtisinghe (1940), proved to be by far the most successful chnique. The reason for its particular success with trout probably lies in the ture of the gut morphology, namely in the extremely thin outer longidinal muscle-coat in the stomach and intestine. This meant that the dye ution, which normally has a poor penetration, could reach the plexus thout disturbance of the nerve arrangement by dissection. Another advantage the presence of the stratum compactum, which forms a strong stable base ıen manipulating the preparations.

During the development of the technique, control experiments wer arranged so that the optimum conditions for each of the following factors wer discovered: fish size; composition of the dye itself and the strength of th solution used; acidity; salinity; time of immersion of preparations in the dy solutions; temperature; fixative and fixation time; clearing agent; use of suc additional substances as hyalase, glucose, sodium acetate.

The final technique used was as follows:

1. Young fish, about 10 cm long, were pithed and the gut dissected out The gut was then cleaned out thoroughly with 0·8% saline solution.

2. The preparations were stretched both evenly and maximally in both circular and longitudinal direction. The most efficient method of achievin this end was by extending large cylindrical pieces of the gut over tapered constricted glass tubes, a whole size-range of which was made. Preparation extended in this way were very easy to handle with the minimum of disturbance. For some regions of the gut it was more convenient to stretch th preparations, muscle-coat uppermost, on a thin wax plate by means of entomological pins. The plates were then placed face down in a dish of dye solution In yet other cases the gut was pumped up with dye solution and sutured.

3. The most effective staining was obtained by placing the preparations i the dye solution immediately after killing the fish. After experiments with refined methylene blue dye types, crude dye (made by Williams and Hopkin i 1913) proved to be the most effective. The dye was made up in a solution o salinity 0·8%. At greater concentration the penetration improved but th nerves disintegrated; when the salinity was less than 0·8%, the nerve-cell only were stained and the contrast was poor. A weak dye solution (0·01%), taking 2 or 3 h to penetrate, produced the most delicate results with th greatest contrast. The acidity of the solution proved to be an important factor The best results were obtained at pH about 5·5 and 6·5 for the stomach an intestine respectively. The solution was maintained at about 12° C durin staining.

4. On completion of staining, the preparations were exposed to the air fo about 4 min in order to oxidize and remove any excess dye, before transferrin them to the fixative, 8% ammonium molybdate being used. In two experiments the fixative containing osmium tetroxide recommended by Alexandrowicz (1951) was used, and gave a better contrast between the nucleus an cytoplasm of the nerve-cells. Kirtisinghe (1940) suggested a duration of 3 min for fixation, but it was found that a fixation time of less than 10 h led t rapid fading of the nerve-fibres.

5. After fixation the gut was cut off the glass tube by a longitudinal incisio and laid out flat. At that stage, the mucous coat could easily be scraped of with a blunt knife down to the stratum compactum. The preparation was the washed with distilled water for 2 to 4 h.

6. The pieces of gut were then gently flattened between two slides and pu in a dish of 95% alcohol for about 1 h, transferred to absolute alcohol for further hour, and then cleared in cedarwood oil. At that stage the stratu

mpactum could be separated off from the muscle-coat so that a thin, flat uscle preparation could be mounted on large slides, longitudinal layer permost.

In most regions the longitudinal muscle-coat is thin, and this, together with e fact that the nerve-arrangement between the two muscle-layers is very mplex, makes further dissection a disadvantage. In the rectum, however, was sometimes an advantage to dissect off the longitudinal coat after the eparation had been in the stain solution for about 1 h. Otherwise the stain d not penetrate to the nerves.

A number of further refinements in methylene blue technology have been ggested in the literature, but the following recommendations have not oved to be particularly successful with trout gut staining. The use of aluronidase as a pretreatment 'spreading factor' (Weddell and Pallie, 1954) moved the staining contrast, the muscle staining blue as well as the nerves. he recommendations of Schabadasch (1934) to increase penetration of the e by means of glucose or pyruvic acid and to improve the staining of fine ucture with sodium acetate and magnesium bromide proved to be of little vantage with the trout gut. The method of heating the saline solution to ° C before adding the dye, when making up the staining solution, suggested Hillarp (1946), produced no specially good results.

Injections of the dye solution during life have not given results of a standard mparable to those obtained by the supravital technique outlined above.

Both the gold chloride (Gairns, 1930) and silver methods (Gunn, 1951; ill, 1927) of preparing whole mounts of the enteric plexus yielded poor sults, owing mainly to heavy staining of the muscle as well as the nerve.

Serial sections. A number of very careful attempts to stain sections of the out gut with silver were made but with no outstanding success, despite ntrolled variations of fixatives, pH, temperature, and times of staining. his supports the view that fresh-water teleosts are particularly refractory to ver staining (Young, 1931).

Both the Bodian (1937) technique using protargol and Holmes's technique olmes, 1947) produced some staining of the subepithelial nerves of the omach and intestine, as well as blackening of the sub-serous region.

The silver technique recommended by Peters (1955) produced by far the ost consistent results. In general the nerve-fibres, especially those of e submucous coat, stained very well but the nerve-cells usually only took e stain peripherally.

The osmium tetroxide technique for staining fixed sections recommended Kiss (1932) produced an even stain of muscle and nerve.

Permanent records were made of characteristic features of the enteric stem by means of photomicrographs. The contrast of methylene blue prerations was found to be optimal for photographic purposes at about three onths, after which time there was a slow fading of the stained parts.

A study was made of the density of nerve-cells present in Auerbach's exus in the oesophagus and in different regions of the stomach and intestine.

Sections of the gut-wall showed that the nerve-cells of Auerbach's plexus lie roughly in one plane, so that counts could be made from the surface. The counts were based on the number of cells contained in sample mm squares. Only selected parts of the best stained preparations were used. Stomach counts were made on preparations taken from 7 fish and intestinal counts from 5. About 5 separate counts were made on each preparation of one region from each fish.

RESULTS

Extrinsic nerves

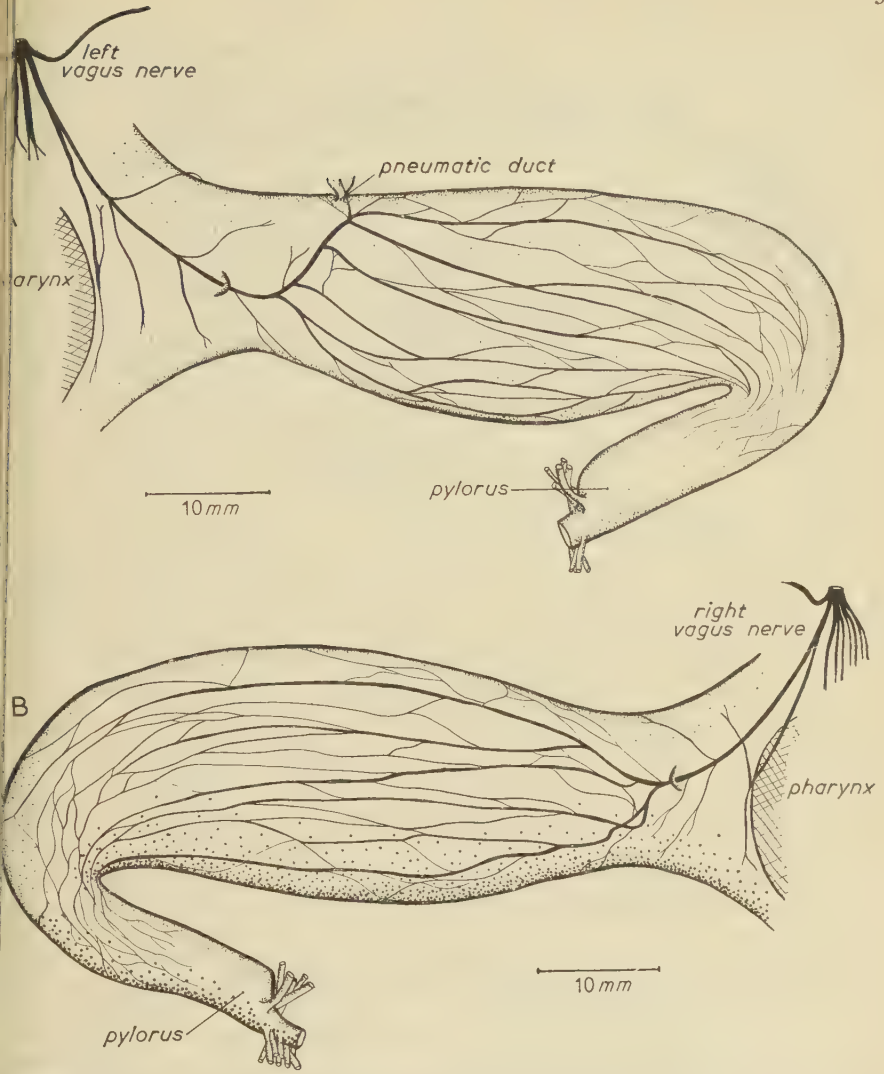
Charts showing the paths of the nerves supplying the gut of the trout have been mapped out from dissections of over 30 fish.

The paths of the vagus nerves are shown in fig. 1. The main vagus trunks penetrate the gut-wall at the oesophagus and run down the stomach between the circular and longitudinal muscle-coats. Two or three branches come off each of the left and right vagus nerves before they enter the gut-wall. These branches contain fibres which innervate the striated muscles of the oesophagus directly. Both vagus nerves run up towards the dorsal surface of the stomach soon after penetrating the gut, the left trunk giving off four major branches ventrally, while three ventral branches come off the right vagus. These branches follow a fairly constant pattern in the first part of their passage between the muscle-coats but the more posterior innervation and minor branch connectives are by no means constant in their arrangement. There are frequent anastomoses between left and right vagal elements, both dorsally and ventrally, although the density of vagus branches is greater on the ventral, inner curvature of the stomach than dorsally. The vagus fibres beyond the antrum are collected in very fine bundles. No fibre bundles have been traced as far as the pyloric sphincter, and it seems most unlikely that any vagus elements extend into the intestine.

Fig. 2 shows the paths of the branches of the coeliaco-mesenteric artery. The splanchnic nerve is very fine and anastomoses during its route along the major branches of this blood-vessel.

Fine left and right branches from the first abdominal sympathetic ganglia join at the junction of the gastric and hepato-intestinal arteries to form a small splanchnic ganglion just anterior to the opening of the pneumatic duct into the right dorsal surface of the oesophagus. There are three main branches from the coeliaco-mesenteric artery at about this level; the gastric and hepato-intestinal arteries and a smaller vessel which supplies the region of the 'duodenal-ileal' junction. Three main splanchnic nerves come off the ganglion to follow these blood-vessels.

The gastric artery and accompanying nerve split up almost immediately in the oesophageal region into left and right branches, which closely adhere to the gut-wall and are embedded in adipose tissue. The left branch supplies the left side of the cardiac stomach and the spleen, while the right artery branches



G. 1. Dorsolateral views of the innervation of the stomach of a trout 228 mm long by (A) left vagus nerve, (B) right vagus nerve.

ain to supply the right side of the cardiac stomach and the pyloric stomach. ne branches of these nerves penetrate the gut-wall at frequent intervals ong the stomach, accompanying minor blood-vessels. Elements from the ht splanchnic gastric nerve join with fibres from both the left and right gus nerves to innervate the pneumatic duct and swim bladder. The lanchnic nerve following the hepato-intestinal artery divides into two anches, one supplying the pyloric caeca and 'duodenum', the other running wn the length of the intestine. A number of the fine splanchnic branches minate in the walls of the arteries and do not reach the gut.

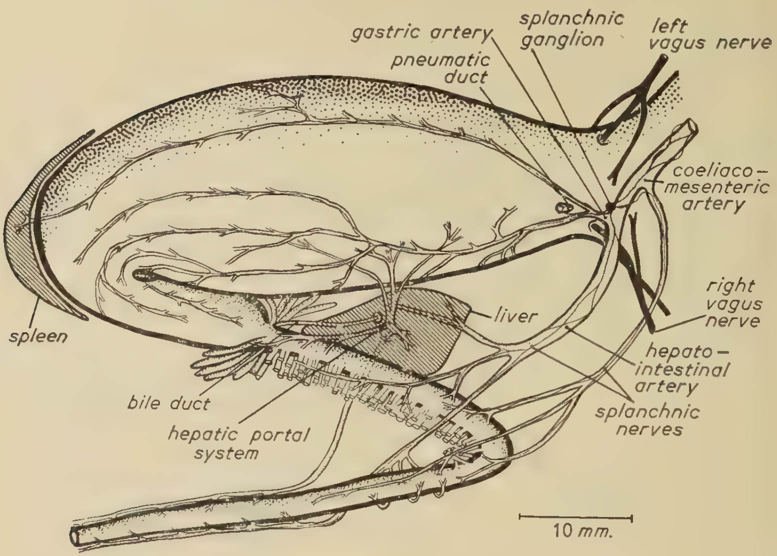


FIG. 2. Path of splanchnic nerve to the trout gut. Fine splanchnic nerves follow all the major branches of the coeliaco-mesenteric artery. The stomach is shown from the dorsal surface.

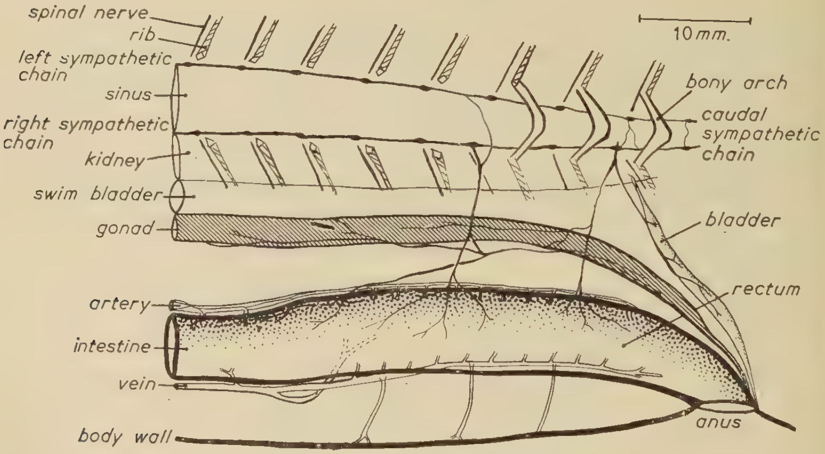
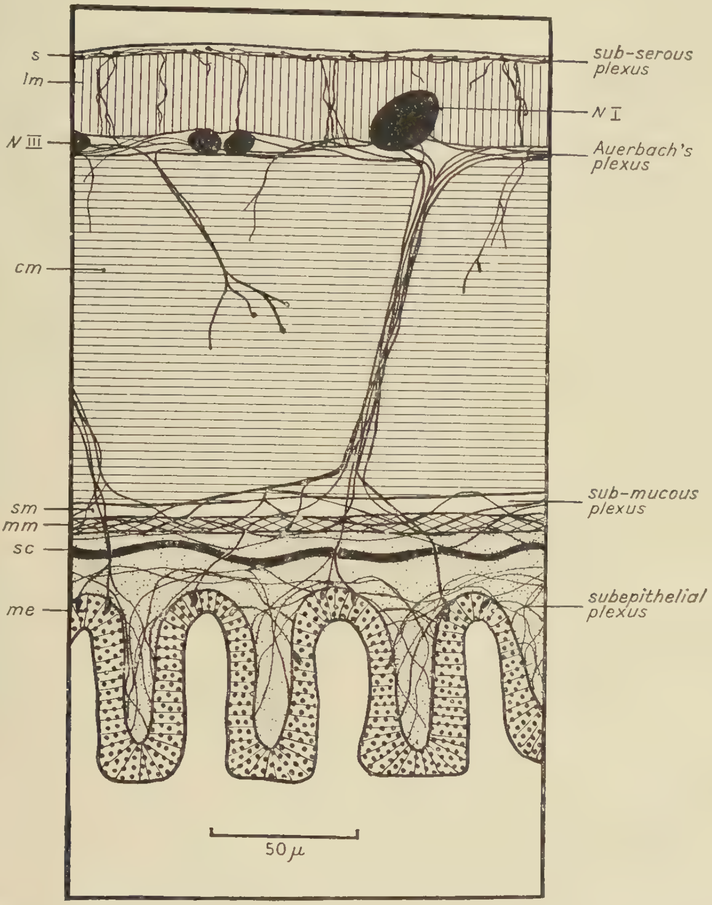


FIG. 3. Innervation of the trout rectum by posterior autonomic nerves.

A posterior 'sympathetic' nerve innervates the rectum as well as the gonads (fig. 3). The nerve originates in the last right abdominal sympathetic ganglion, but an extremely fine nerve joins it close to this ganglion from the left sympathetic chain. Two segments farther back another nerve penetrates the kidney and swim bladder to supply the gonads, urinary bladder, and rectum. It was not possible to see whether this nerve originated from the sympathetic chain or from spinal nerves.

teric plexus

Jeneral plexus structure. The distribution of nervous elements in the gut-
ll is shown diagrammatically in fig. 4.



4. Diagrammatic transverse section through the wall of the trout stomach, to show the
eral arrangement of nervous elements. Compiled from both silver and methylene blue
ied preparations. *cm*, circular muscle; *lm*, longitudinal muscle; *me*, mucosal epithelium;
muscularis mucosa; *N I*, *N III*, types I and III nerve-cells; *s*, serosa; *sc*, stratum compactum; *sm*, sub-mucous coat.

uerbach's plexus lies prominently between the longitudinal and circular
scle-coats. Throughout most of the gut there are no true ganglia in the
xus since only from 1 to 3 nerve-cells occur at the nodes and many cells lie
gly along the internodal connectives (fig. 5, A). A smaller number of large
ve-cells is embedded in the longitudinal muscle, their axons passing down
oin the plexus (fig. 5, B). A third cell type, which is clearly associated with
al fibres, is found only in the oesophagus and stomach.

Bundles of fibres pass down through the circular muscle at irregular intervals from Auerbach's plexus to join a prominent and loosely arranged plexus of nerve-fibres which lies between the stratum compactum and the circular muscle-coat. This plexus is present in the intestine as well as in the stomach, despite the absence of a sub-mucous coat and muscularis mucosae in the former. It occupies a similar position to Meissner's plexus of mammals, but no nerve-cells are present. A complex subepithelial plexus is present. It is very prominent in the stomach, some of the fibres ending about glandular cells while others pass at frequent intervals through the stratum compactum to join the 'sub-mucous' plexus. In the intestine considerably fewer fibres are to be seen subepithelially and the stratum compactum is only rarely penetrated by nerves. Structures embedded in the epithelium, especially at the bases of the villi, stain strongly with silver and appear to be connected to the subepithelial plexus. A few small, poly-axonal nerve-cells have been seen in the subepithelium.

A complex arrangement of cells staining with methylene blue lies between the muscle-coats, slightly embedded in the longitudinal muscle; it is shown diagrammatically in fig. 6, A. Processes appear to penetrate between the muscle-fibres. This arrangement bears a close similarity to descriptions of the 'interstitial cell network of Cajal' seen in other vertebrate enteric systems.

A sub-serous nerve plexus, which is particularly prominent in the oesophagus and rectum, is formed of a meshwork of extremely fine fibres, connected with roughly spherical masses, 1–4 μ in diameter, embedded in the serous coat. Some of the fibres run down through the longitudinal coat to join the myenteric plexus.

The main branches of the splanchnic nerve run superficially along the gut. Minor branches, following the course of the smaller blood-vessels, penetrate the serous coat and longitudinal muscle-layer along the whole length of the gut to join Auerbach's plexus (fig. 6, B). Sometimes fine fibres in the splanchnic branches pass to the sub-serous plexus. Other fibres appear to pass directly to the smooth muscle-coats.

The main branches of the vagus nerves run down the stomach between the muscle-coats. From these trunks minor branches are given off which, besides anastomosing with each other, soon merge intimately with the enteric plexus proper (fig. 6, C). Pericellular endings of vagal fibres are found about enteric neurones.

Nerve-cells. Three main types of nerve-cells have been distinguished in Auerbach's plexus, both by their structure and distribution (fig. 7).

Large (30–60 μ diameter), multipolar cells in the outer region of Auerbach's

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FIG. 5 (plate). A, photomicrograph of a methylene blue 'whole mount' preparation of the posterior cardiac stomach. Auerbach's plexus can be seen lying between the muscle-coats. Type III nerve-cells lie at the nodes and along the internodal connexions.

B, photomicrograph of a methylene blue preparation of the intestine. It is focused so that the type I nerve-cells embedded in the longitudinal muscle can be seen clearly, while the plexus proper formed from type III cells is just visible in the background.

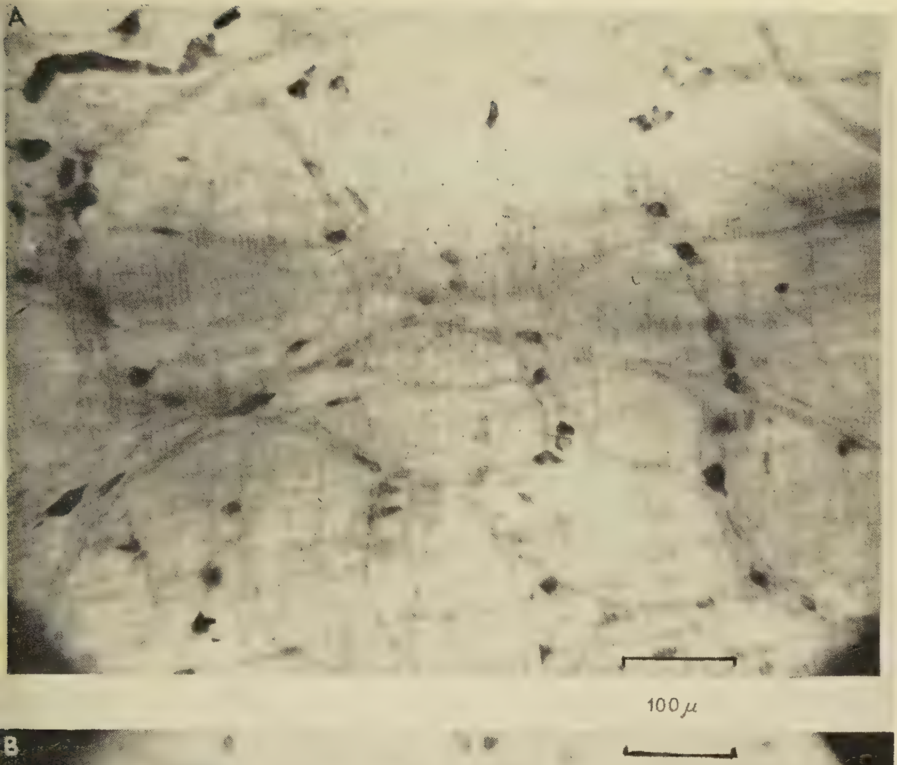

FIG. 5

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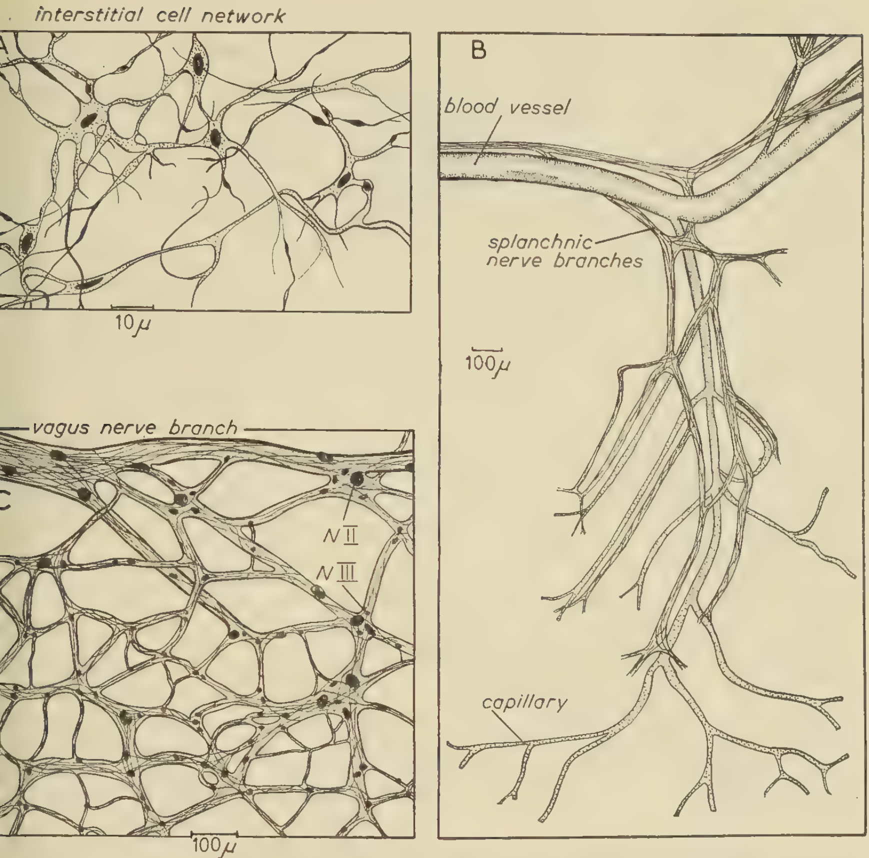

G. 6. A, diagram showing the network staining with methylene blue which lies in close ation to the longitudinal muscle-coat and probably represents the 'interstitial network of jal'. B, diagram showing a typical small splanchnic nerve following the course of minor eries to penetrate the longitudinal muscle-coat and join Auerbach's plexus. Surface view methylene blue whole mount. Minor branches have penetrated through the transparent eared) longitudinal muscle-coat. C, diagram showing the integration of branches of the vagus nerve with Auerbach's plexus in the mid-cardiac stomach.

exus lie half-embedded in the longitudinal muscle along the whole length of e gut (fig. 7, A). Their axons, which possess a few short collateral branches, ss down to join the main body of the plexus. There are numerous short anching dendrites, which have never been seen to end on other nerve-cells the same or of different type. No pericellular endings have been seen on the ll-bodies of these type I cells.

Large cells, which are usually to some extent elongate (30–50 μ × 60–90 μ), between the circular and longitudinal muscle-coats. The nucleus is atively small, with a prominent nucleolus. These type II cells (fig. 7, B) ow a gradual transition of form from the oesophagus to the pylorus, but ey have not been seen beyond this point. Large aggregations of type II cells, netimes as many as 30, are found at the points of entry of the vagus nerves into the oesophagus. These cells are nearly always pear-shaped and monopolar. Many more cells lie along the vagus branches and a number join cells of type III to form large diffuse ganglia in the oesophagus and anterior part of the stomach. These are often elongate, bipolar cells, or monopolar cells in which the process divides into two soon after leaving the cell. Towards the posterior part of the cardiac stomach, and even more frequently in the pyloric stomach, the cells have two or three long dendrites whose branches end in delicate boutons. The axon possesses collaterals. These posteriorly placed type II cells are sometimes difficult to distinguish from type I cells. It is on nerve-cells of type II that pericellular endings of vagal fibres are found.

Type III neurones are small cells (10–15 μ in diameter) with very large nuclei which do not take the stain (fig. 7, C). They form the main body of Auerbach's plexus between the muscle-coats, where they lie both at the nodal points and along the internodal connectives. Cells of type III may be uni-, bi-, or multi-polar. Uni- and bi-polar cells are found alone, mainly at minor junctions in the plexus, their axons passing down into the circular muscle, while multipolar cells with up to 5 short branching dendrites are found at the larger nodal junctions, where other cells are present. In some preparations the dendrites appear to lie on the cell bodies of adjacent cells.

Nerve endings. Fine, smooth vagal fibres run alongside individual striated muscle-cells in the oesophagus. They give off from 3 to 6 branches, which divide further to end in fine clumps on the surface of the muscle, over a distance of 100 to 350 μ. This arrangement is shown diagrammatically in fig. 8, A. It was not possible to see the relation of the nerve-fibres to the sarcoplasm.

Nerve-cells of type II in the cardiac stomach are commonly found to be enveloped by from 1 to 4 fine, intertwining, varicose fibres, which in a number of cases have been traced back to vagal trunks (figs. 8, B, C.). Not all the varicose fibres terminate on the cell surface. Some pass on to rejoin the plexus and end on other type II cells. Some of the cells are enveloped by a capsule on which the intertwining fibres lie, rather than on the cell membrane.

Most of the axons of all the enteric neurones and the dendrites of type II and III cells run into the plexus and cannot be traced very far. Fine fibres separate from Auerbach's plexus and run down to end in the circular muscle-coat. They terminate, after branching, in small, heavily stained swellings or boutons. However, like most previous workers who have used silver and methylene blue staining techniques, I have not been able to see the precise nature of the relation between the nerves and individual smooth muscle-cells.

Nerve-endings are present in the mucosa. In the stomach, fibres separate off from the subepithelial plexus and surround the gastric gland cells. Heavily

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FIG. 7 (plate). Photomicrographs of nerve-cells.

A, type I nerve-cell embedded in the longitudinal muscle-coat. Note the short dendrites and the collateral branches coming off the axon.

B, type II nerve-cell, associated with the vagal nerve. Note the long dendrites.

C, type III nerve-cells, forming the plexus proper. Note the relatively small size and the prominent, lightly staining nucleus.

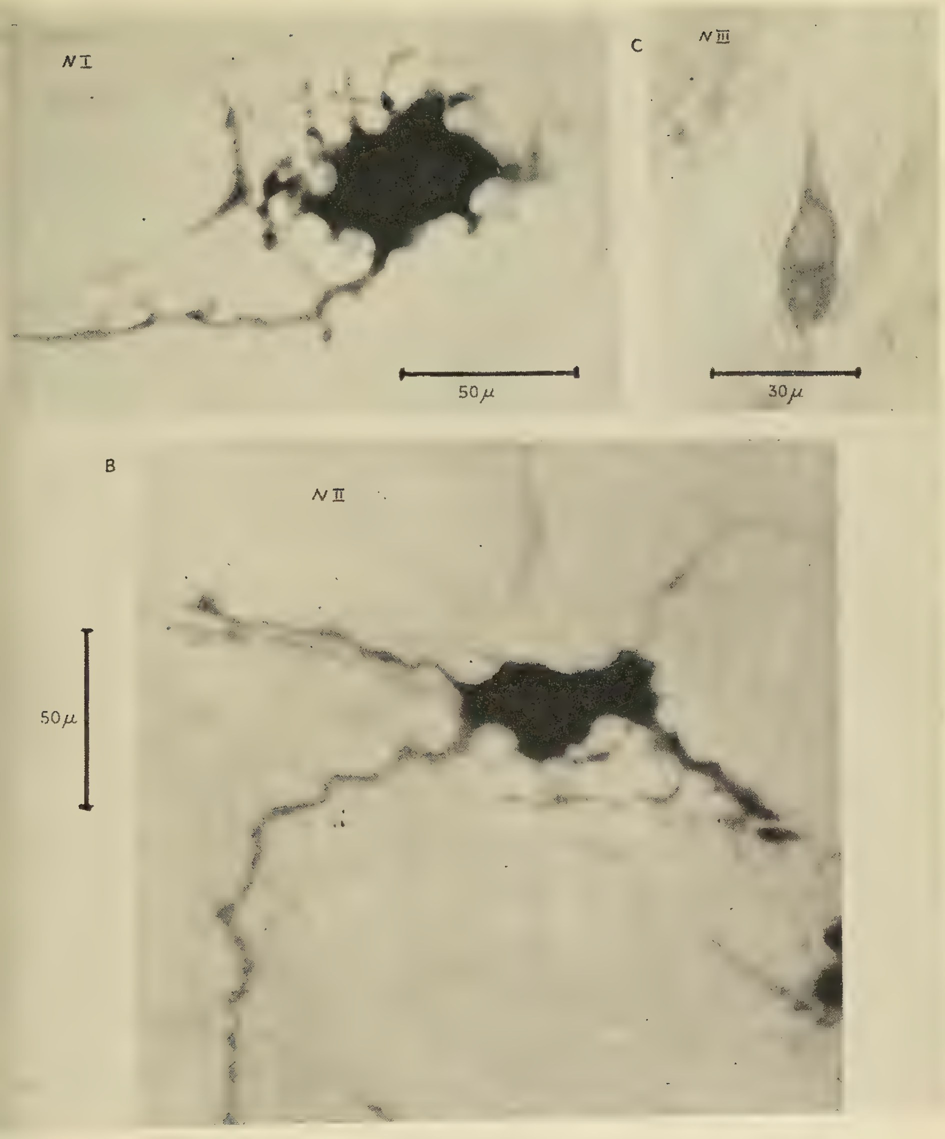
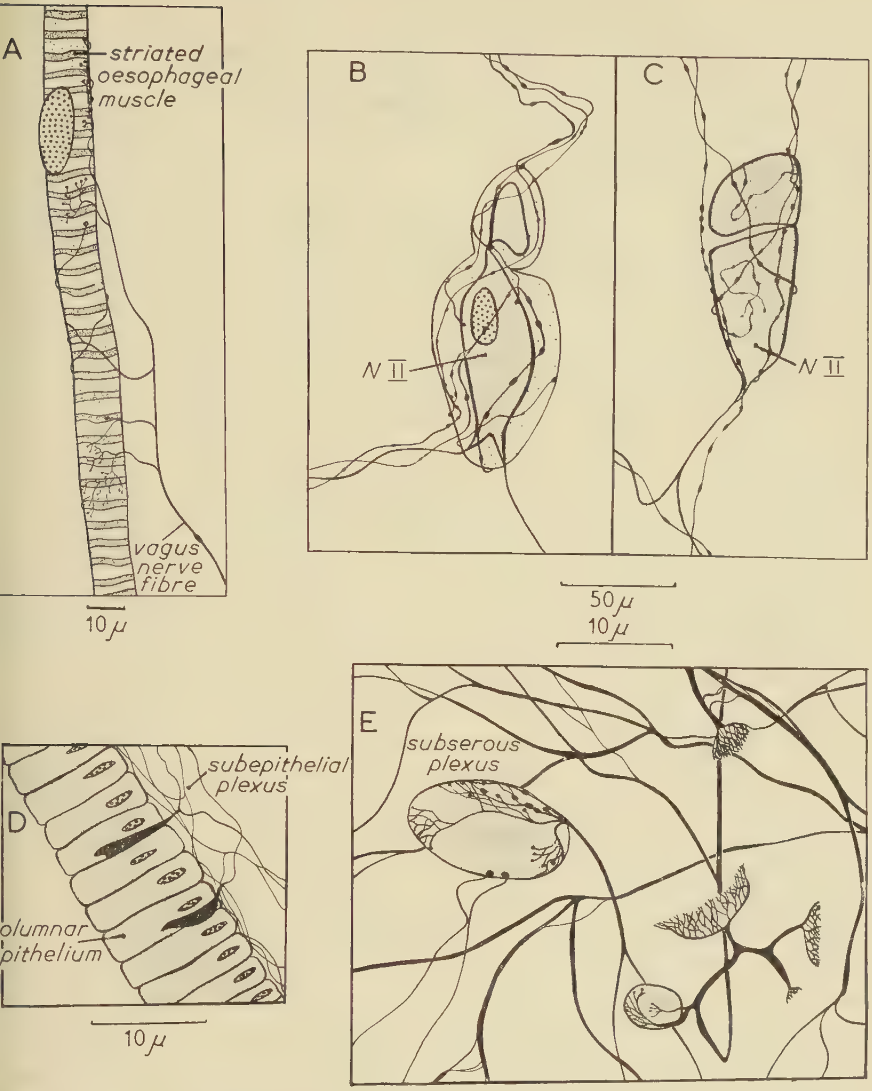


Fig. 7

G. BURNSTOCK



G. 8. A, diagram showing the innervation of a striated oesophageal muscle by a vagal fibre. C, pericellular endings of vagal fibres about type II nerve-cells (*N II*). A capsule envelops neurone shown in B. D, diagram showing structures lying between the bases of the columnar ithelial cells, from which fibres run to the subepithelial plexus. E, diagram showing the form the sub-serous plexus under high magnification, and its connexion with structures embedded in the serous coat.

aining structures lie towards the bases of the epithelial cells of the intestine, pecially at the proximal ends of the villi. They appear to be connected with ores of the subepithelial plexus (fig. 8, D). In addition, fibres are often seen to n along the bases of the epithelial cells. Whether they form part of local reflex arcs or whether they pass directly to higher centres by way of the extrinsic nerves cannot be decided from the preparations.

Fine fibres of the subserous plexus divide arboreally to end in very delicate boutons usually on the surface of small (3–10 μ diameter) ovoid structures (fig. 8, E). These endings seem to be confined to parts of the rectum and to the anterior oesophagus.

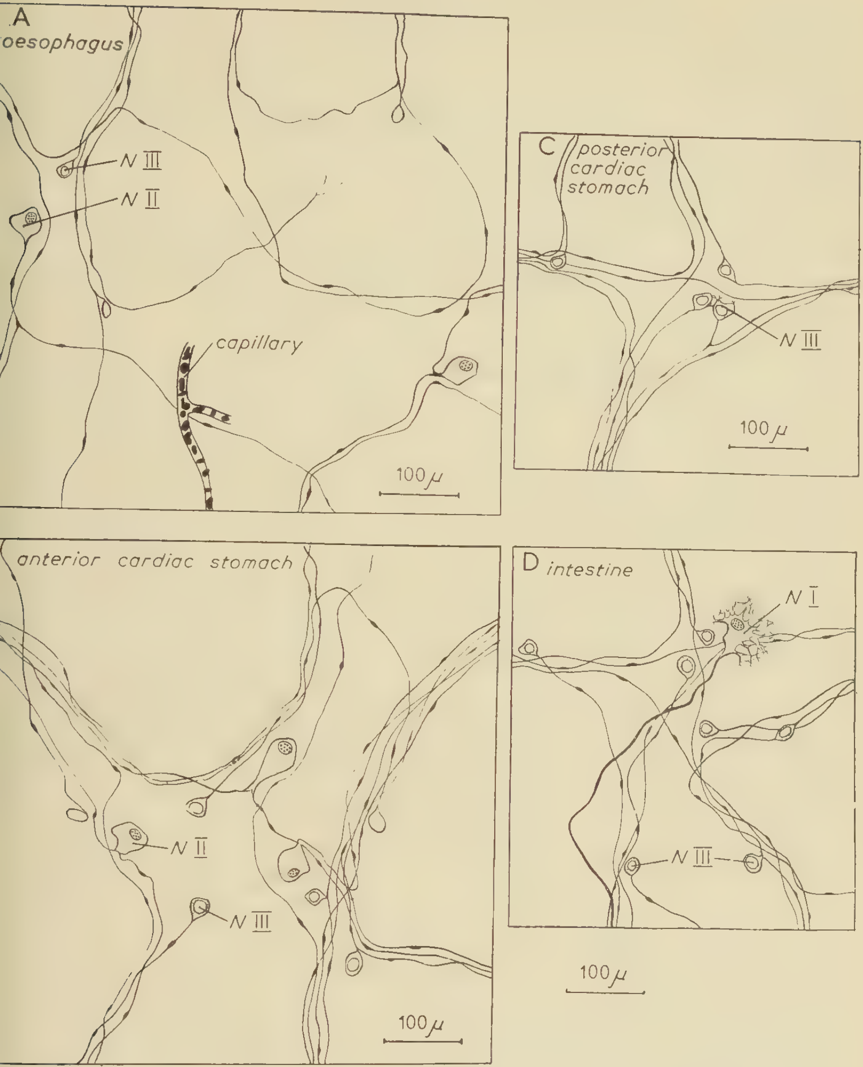
Regional analysis. The plexus is very diffuse and heterogeneous in the oesophagus. The nodal points sometimes contain up to 8 or 9 nerve-cells (types II and III) while the interganglionic connectives are long and contain only a few fibres (fig. 9, A).

The vagus nerves enter the gut in this region. Aggregations of type II nerve-cells are found along the vagus trunks, both before and after their entry into the gut. A number of fine side branches of the vagus nerves are given off in the oesophagus. These become incorporated in the beginnings of the enteric plexus.

Fine branches of the splanchnic nerve enter the oesophageal wall, very close to the right vagus nerve, and contribute directly to the plexus. No nerve-cell aggregations are connected with the entry of the splanchnic branches. No type I nerve-cells have been seen in the oesophagus, perhaps because no smooth muscles are present. The subserous plexus is particularly prominent in this region. The plexus in the anterior cardiac stomach is similar to that in the oesophagus, with nodes containing up to 8 nerve-cells, from which 5 to 8 irregular inter-ganglionic connectives radiate (fig. 9, B). From the mid-cardiac stomach to the antrum, however, the plexus becomes progressively more homogeneous and more densely packed. The nodal points become much smaller with only from 1 to 4 nerve-cells involved (fig. 9, C). The internodal fibres are much shorter and only from 3 to 5 radiate from each nodal point. Where nerve-cells lie along internodal connectives, one or two fibres come off at this point and pass down into the circular muscle. Nerve-cells of type III almost exclusively form the plexus proper towards the posterior part, but type II cells are seen in the more anterior nodes and at points where the vagus branches merge with the plexus. Further type II cells bearing pericellular nerve-endings are present in the internodal spaces. Type I cells are found in this region, embedded in the longitudinal coat.

The subepithelial plexus is very prominent. Fibres from this plexus frequently end round the glandular cells. In the pyloric stomach the plexus itself does not appear to be so dense as that found in the posterior cardiac stomach and the antrum. It is formed largely of type III nerve-cells at the nodal points. Type I cells are fairly prominent in this region and type II cells with long branching dendrites are frequently present. Branches of the vagus nerves, although now fine and intimately related to the plexus proper, pass down the pyloric stomach, but no vagus elements have been traced as far as the pyloric sphincter. Splanchnic fibres to the pyloric stomach are supplied mainly from branches coming off the right gastric nerve.

Auerbach's plexus is very dense in the intestine. One to three nerve-cells of



9. Form of Auerbach's plexus in different regions of the gut. Same scale throughout. iagram of the diffuse arrangement of the myenteric plexus seen in the *oesophagus*, with ted nerve-cells of types II and III (*N II* and *N III*). B, typical large ganglion-type node aining both type II and III nerve-cells, as found in the *anterior cardiac stomach*. C, cal small node containing about 3 nerve-cells of type III (*N III*), as found in middle and *rior cardiac stomach* and pyloric stomach. A single nerve-cell can be seen lying along an nodal connective. D, diagram of the characteristic arrangement of type III nerve-cells *II*) found in the myenteric plexus of the *intestine*. The axon of a type I nerve-cell can be seen joining the plexus proper.

e III occupy the nodal points and some lie along the short internodal nectives (fig. 9, D). Nerve-cells of type I are present. The plexus lying veen the mucosa and the circular muscle is prominent in the intestine and many fibres run through the circular muscle to join the myenteric plexus The subepithelial plexus, however, is not prominent. Extrinsic control i mediated entirely by branches of the splanchnic nerve. This region, especiall more posteriorly, is highly vascular and many blood-vessels follow the comple pattern of the nerve-plexus. The pyloric caeca contain a continuation of th intestinal nerve-plexus between the muscle-coats.

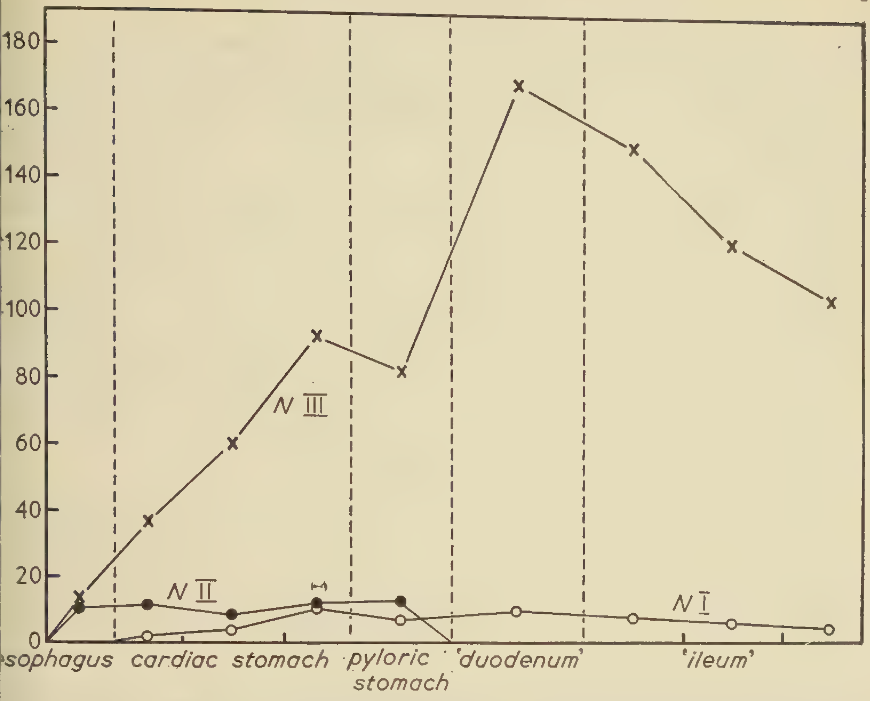
Preparations of the rectum were poor. They did show, however, that th plexus is more evenly arranged than that seen in the more anterior regions with fairly regularly shaped inter-plexus spaces. The cells at the nodal point and along the connectives are all of type III. Cells of type I are present. Th subserous nerve-plexus is prominent in the rectum.

Table 1 summarizes the results of regional nerve-cell counts. The figure showing the number of nerve-cells per square mm for each fish represent th average taken from 2 to 5 counts made on each regional preparation. Separat counts of nerve-cells of all 3 types were included in the calculations. Preparations from the following regions were used: oesophagus, anterior, middle an posterior cardiac stomach, pyloric stomach, 'duodenum' (defined for convenience as the anterior part of the intestine from which the pyloric caec arise), anterior, middle, and posterior 'ileum' (intestinal region between 'duodenum' and rectum). At the foot of the table the average number o nerve-cells of types I, II, and III per square mm found in each region of al the fish used is given. These figures are represented graphically in fig. 10.

Type I cells first appear in the anterior cardiac stomach and rise to density of 11 cells per square mm at the posterior cardiac stomach, from which there is a gradual decline towards the rectum. There is a definite correlation of cell I density with that of cell III, i.e. there is one type I cell to approximately 20 type III cells.

Type II cells show a fairly constant density of about 10 cells per square mm throughout the oesophagus and stomach. They are not present in the intestine The distribution of these cells is not even, however, especially in the oesophagus, where the figures reveal a variation of from 1 to 31 cells per square mm in areas taken at random. The explanation is that most of the cells are aggregated about the vagal trunks, so that areas counted of the plexus prope contain very few type II cells.

Type III cells are present in much greater numbers than those of the othe two types. Furthermore, they show a definite density gradient. There is gradual increase from 14 to 93 cells per square mm from the anterior to posterior cardiac stomach. This density drops very slightly in the pyloric stomach before rising to 170 cells per square mm in the duodenum. From th duodenum there is a gradual density fall to 105 cells per square mm at th posterior ileum. There are two nerve-cell density peaks, one at the antrum an one in the duodenum. It is interesting to note that in the living system, many movements of the stomach first appear at the antrum. In the intestine, peristaltic waves of contraction move down the gut from a point about one-thir of the way down the duodenum.



. 10. Graph showing the density of nerve-cells of all three types (*N I*, *N II*, and *N III*) in various regions of the gut. The average number of cells per square mm of the different ions was taken from table 1 (p. 220), which was compiled from 64 stained preparations made from 11 fish.

The total number of nerve-cells in the trout gut has been calculated to be proximately half a million.

Discussion

trinsic nerves

In the trout, the vagus nerve does not extend beyond the stomach into the estine. This result supports the findings of Young (1931) for the stomach *Uranoscopus* and *Lophius*, although unlike these teleosts the right vagus of trout is not longer than the left. Pericellular endings of the pre-ganglionic gal fibres end about type II nerve-cells. Similar endings were found to velop neurones in the stomach of *Motella* (Kirtisinghe, 1940). Neither type cells nor vagal fibres are found posteriorly (caudally) to the stomach. The g latent period before the longitudinal stomach muscles contract on nulation of the vagus nerve (Burnstock, 1958*a*) supports the morphological dence for the presence of synapses between preganglionic vagal fibres and irones in the gut-wall. In addition both nicotine and hexamethonium, drugs ich block the ganglia, eliminate the response to vagus stimulation (Burnck, 1958*b*).

Some of the vagal fibres directly innervate the striated oesophageal muscles of the trout and on stimulation of the vagus nerves these muscles contrac rapidly some seconds before the smooth stomach-muscle responds. It i interesting, however, that the oesophageal muscles are capable of sustaine contraction if the stomach is extended with food, even after the vagus nerve have been severed.

Chevrel (1887) and Young (1931) showed that the 'sympathetic' innervation of the teleost gut consists of a single anterior splanchnic nerve, branche of which closely follow the main arteries supplying the gut. In the trout, too, very fine splanchnic nerve supplies the stomach and intestine. The response o the stomach to stimulation of the splanchnic nerve is rapid, in contrast to th delayed reaction on vagus stimulation (Burnstock, 1958*b*). This fact support the morphological evidence that most of the splanchnic fibres reaching th gut are postganglionic and innervate the muscles directly. Thus vagal an 'sympathetic' innervation of the teleost gut corresponds to that described i mammals (Dogiel, 1899; Kuntz, 1922; Lawrentjew, 1931).

Separate autonomic innervation of the hind gut is present in the trout contrary to the findings of Chevrel (1887) and Young (1931) working o other teleosts. This discovery has been given physiological support in previous paper, where the result of electrical stimulation of these nerves i described (Burnstock, 1958*b*). The anterior of the two nerves is clearl 'sympathetic', originating in the last abdominal ganglia, but whether the othe nerve contains 'sympathetic' or 'parasympathetic' fibres is not clear fro the dissections. It is conceivable that posterior autonomic control has evolve in teleost fish in relation to the specialized development of separate urinar and reproductive systems. Alternatively it may have developed in relation t the complicated rectal septum apparatus described elsewhere (Burnstock 1959), the function of which is not yet clear.

Enteric plexuses

The nerve-cells present in the trout gut lie diffusely in the meshes o Auerbach's plexus throughout most of its length and are not aggregated int ganglia, as is the case in mammals (Kuntz, 1953; Hill, 1927). In this respec the form of the trout plexus resembles that of other teleosts described b Sakussef (1897) and Kirtisinghe (1940), as well as those found in the amphibians (Gunn, 1951) and elasmobranchs (Kirtisinghe, 1940).

In this paper, nerve-cells forming the trout myenteric plexus have bee classified into three distinct types by their distribution as well as by thei structure. Despite the general lack of agreement about enteric nerve-ce classification, it is clear from descriptions and pictures that these cells are ver similar to those described in other teleosts (Kirtisinghe, 1940) and amphibian (Gunn, 1951). Type III cells, however, which form the major part of th plexus proper in the trout, do not appear to be represented in the mammalia gut.

Embryological studies have shown that a number of enteric neurone migrate down the vagal trunks at a late stage in gut development (Kuntz

920; Van Campenhout, 1941). The pear-shaped type II cells seen along the agal nerves are less complex than those cells of the same type with branching lendrites found in the meshes of the plexus in the pyloric stomach. Type II ells are clearly concerned with vagal control. Pericellular endings of preganglionic vagal fibres are found to envelop a large number of type II cells. These cells may be the vagal motor neurones, or they may represent a third eurone in the peripheral 'parasympathetic' pathway, their axons each supplyng a large number of motor neurones, a suggestion originally put forward heoretically by Langley (1922).

Type III cells probably form the basic connective, motor, and inhibitory eurones of the enteric system. That elements with these functions may exist n the trout gut has been demonstrated (Burnstock, 1958*b*). Because type I cells are clearly associated with the vagus nerves, and type I cells are few n number with their dendrites ending in longitudinal muscle only, cells of ype III are the neurones most likely to be concerned with this function. In ddition axons of type III cells have been seen to innervate the muscles.

Type I cells may be sensory. Their dendrites are embedded in the longiudinal muscle and might be concerned with recording stretch. The existence of sensory neurones involved in the peristaltic reflex arc seen in mammalian gut has been clearly demonstrated recently (Bülbring, Lin, and Schofield, 1958). Alternatively they may represent 'pacemaker' cells, one type I cell controlling o type III cells. Keith (1915) put forward the suggestion that the gut may ossess intestinal 'pacemakers' by analogy with the sinu-atrial node of the eart.

The existence of a sub-mucous plexus has been reported previously in the eleost gut (Monti, 1895; Sakussef, 1897), but unlike Meissner's plexus, so haracteristic of the mammalian sub-mucous coat (Stöhr, 1932), no nerve-cells re present. A similar condition has been described for Amphibia (Gunn, 951).

The sub-epithelial plexus, also found in mammals (Schabadasch, 1930), is particularly prominent feature of the trout gut, and small polyaxonal cells imilar to those described in the tench by Monti (1895) are present. No such ells have been reported in any other vertebrate class.

As in other vertebrate classes (Dogiel, 1896; Hill, 1927; Gunn, 1951), ensory endings in the mucosal epithelium have been identified in the trout ut. Capparelli (1891) described sensory endings in the mucosal epithelium f Amphibia, which are very similar to those observed in the trout. Sensory ndings of a rather different nature have been described in the epithelium of he perch by Sakussef (1897). Whether they form part of local peristaltic eflex arcs in the manner suggested by Bülbring, Lin, and Schofield (1958) or whether they connect directly to higher centres by way of extrinsic nerves annot be decided from the preparations.

A subserous nerve plexus has been described in the gut of mammals Worobiew, 1913; Kondratjew, 1928; Schabadasch, 1930). Although not reviously reported in fish, this plexus exists in the trout gut, where it is prominent in the oesophagus and rectum. The tiny clumps embedded in the serous coat and connected to the plexus bear a very close resemblance to the encapsulated tactile nerve-endings in teleost skin described by Weddell and Pallie (1954). If indeed these structures are sensory, further investigation of their precise function would be interesting.

A structure resembling descriptions of the 'interstitial network of Cajal' is also present in the trout gut. This is a feature which appears to be common to all vertebrate enteric systems (Hillarp, 1946; Meyling, 1953). In the trout gut, however, it seems to be connected with the longitudinal muscle-coat only.

Studies of the numbers of nerve-cells present in the enteric plexus of the trout reveal density gradients with peaks in the antrum and anterior 'duodenum'. No quantitative studies of this kind have been done on any other lower vertebrates. Studies of the mammalian gut, however, show that there are density peaks at the pylorus and anus of the guinea-pig (Irwin, 1931). The actual density of nerve-cells present in the myenteric plexus of the trout (100 to 200 cells per square mm) is about the same as that reported in mammals (Irwin, 1931; Ohkubo, 1936). However, the total number of cells calculated to be present in the trout gut (about half a million) is considerably less than the figure given for mammals (5 million in the small intestine of the cat (Sauer and Rumble, 1946)). Prominent peristaltic waves are initiated where there are nerve-cell density peaks, i.e. at the antrum and one-third of the way down the duodenum. This result supports Alverez's 'gradient' theory (Alverez, 1927) for the mechanism of peristalsis, but a reflex action also seems to be involved in the trout. Experiments with short segments of the stomach suspended under tension to record circular muscle-contractions, still exhibit regular waves of constriction moving away from the area of greatest nerve density. Thus it seems that while peristalsis is basically a reflex action, the first circular contraction occurs at the point of greatest cell density in any one segment, while the direction of the wave is normally dictated by the direction of the neurone density gradient. Thus although the cardia, the antrum, and the region one-third of the way down the 'duodenum' may be called the 'pacemakers' of the peristaltic waves seen in the trout gut, they need not possess any special feature other than the peak nerve density values of the gradient.

Fig. 1 has been reproduced from the *Journal of Physiology* by kind permission of the Editorial Board.

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TABLE 1. *The table shows the number of nerve-cells of each type (I, II and III) per square mm in each part of the alimentary canal*

Fish	No. of preparations					Stomach																Intestine															
		Oesophagus				*Ant. cardiac*				*Mid. cardiac*				*Post. cardiac*				*Pyloric stomach*				*'Duodenum'*				*Ant. ileum*				*Mid. ileum*				*Post. ileum*			
		I	*II*	*III*	*Total*	*I*	*II*	*III*	*Total*	*I*	*II*	*III*	*Total*	*I*	*II*	*III*	*Total*	*I*	*II*	*III*	*Total*	*I*	*II*	*III*	*Total*	*I*	*II*	*III*	*Total*	*I*	*II*	*III*	*Total*	*I*	*II*	*III*	*Total*
1	12	0	2	6	8	2	14	29	45	4	7	49	60	12	21	87	120	6	15	74	95	12	0	78	190	9	0	171	180	6	0	114	120	6	0	106	112
2	8	0	31	14	45	1	6	33	40	4	2	69	75	8	13	114	135	4	17	84	105																
3	4	0	12	13	25	3	7	42	52	4	9	70	83	6	2	87	95																				
4	5	0	1	8	9																	9	0	171	180	6	0	159	165	8	0	146	154	6	0	114	120
5	10	1	16	16	33	1	21	51	73	3	23	52	78	14	19	97	130									7	0	179	186	9	0	156	165	6	0	115	121
6	8	0	23	19	42	0	12	20	32	5	5	88	98									10	0	182	192	8	0	137	145	6	0	136	142	4	0	94	98
7	2	0	1	13	14	1	9	58	68	4	13	67	84	9	7	84	100																				
8	4																	8	9	78	95					8	0	122	130	5	0	101	106	5	0	95	100
9	6					2	15	34	51	2	6	57	65	21	17	105	143	8	9	91	108									5	0	73	78				
10	3	0	2	19	21	2	12	29	43	4	9	39	52	5	4	75	84																				
11	2																					8	0	144	152	7	0	140	147	6	0	127	133				
Means		0	11	14	25	2	12	37	51	4	9	60	73	11	12	93	116	7	13	82	101	10	0	170	180	8	0	151	159	6	0	122	128	5	0	105	110

Spermatophore Production in *Rhodnius prolixus*

By K. G. DAVEY

(*From the Department of Zoology, University of Cambridge; present address: Department of Zoology, University of Toronto*)

With one plate (fig. 5)

SUMMARY

On the basis of experimental and histochemical evidence it has been shown that the main body of the spermatophore of *Rhodnius* is a mucoprotein or neutral mucopolysaccharide originating in the transparent accessory glands of the male. A study of the events during copulation demonstrated that the spermatophore is formed inside the spermatophore sac, the folded terminal portion of the intromittent organ. Normally folded inside the base of the penis, the spermatophore sac is everted by the influx of the transparent secretion and acts as a mould for the spermatophore. The solidification of the fluid, transparent secretion inside the sac is the result of a lowering in pH brought about by the secretion of the bulbus ejaculatorius.

INTRODUCTION

DURING copulation the males of a number of insects enclose the semen in a spermatophore, the product of the male accessory glands, before transferring the whole structure to the female. Some spermatophores have been described, and in a few cases the process of formation has been investigated, but a satisfactory explanation of the mechanism by which the fluid secretion of the accessory glands is converted to the solid material of the spermatophore has not emerged from these studies. This paper presents results which reveal the processes involved in the formation of the relatively simple spermatophore in the large blood-sucking bug, *Rhodnius prolixus*.

RESULTS

The spermatophore in Rhodnius

The spermatophore of *Rhodnius* has been described in some detail by Khalifa (1950). Essentially it is a transparent, homogeneous, gelatinous, pear-shaped mass with a slit containing the semen in the dorsal surface of the anterior end (fig. 1). There is a slight opacity just posterior to the slit; this is the secretion of the opaque accessory glands of the male and its function is described elsewhere (Davey, 1958). In the female, the slit encloses a muscular pad on the dorsal surface of the bursa copulatrix, thereby holding the semen against the vestibulum of the oviduct. For details of the structure of the female tract the reader is referred to papers by Galliard (1935) and Khalifa (1950).

The male genitalia

In order to understand the events at copulation leading to the formation of the spermatophore, it is necessary to examine the structure of the external

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genitalia of the male. The heteropteran intromittent organ is contained in the genital capsule (fig. 2), the floor of which is a heavily sclerotized cup variously interpreted as the remains of the ninth sternite (Snodgrass, 1935) and as the fused gonocoxopodites (Bonhag and Wick, 1953). On the dorso-lateral borders of the capsule is a pair of claspers. These are referred to as gonostyli by Bonhag and Wick (1953), but recent work by Snodgrass (1957) indicates that

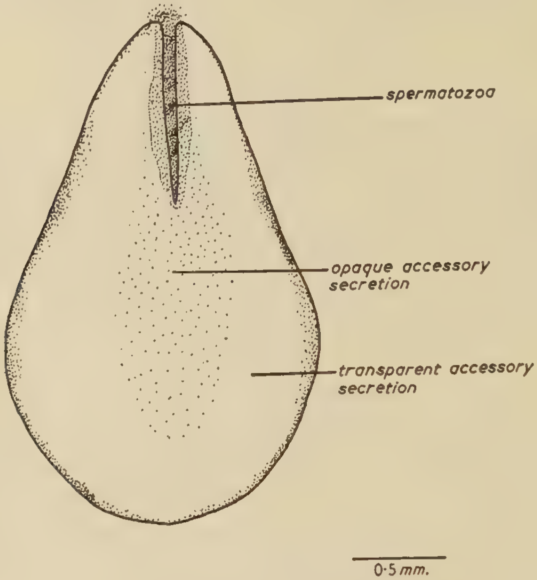


FIG. 1. A spermatophore of *Rhodnius*, dorsal view.

they are more likely homologous with the parameres, or lobes of the ectodermal outgrowths from which the phallic organs develop. The roof of the genital capsule is formed by the anus and its associated sclerites. The terminal segments have been extended in fig. 2; normally the genital capsule lies telescoped within the horseshoe-shaped eighth sternite and the ring-shaped seventh sternite.

The nomenclature of the various parts of the genitalia of insects is confused, but an attempt has been made in this study to adhere to the system of Snodgrass (1935). Potash preparations of the intromittent organ of *Rhodnius* are not particularly revealing and the external morphology of the genitalia can be seen in the photograph in fig. 5, A (opposite p. 225). The phallobase is a saddle-shaped sclerite with two long basal apodemes as figured by Singh-Pruthi (1925) for other Reduviidae. The aedeagus appears to consist of a heavily sclerotized plate on the dorsal surface with rather more membranous ventral and lateral surfaces.

A longitudinal section of the terminal segments of the male reveals a more complicated situation (fig. 3). The ejaculatory duct opens into a much-folded internal sac, the lumen of which has been stippled and which has been

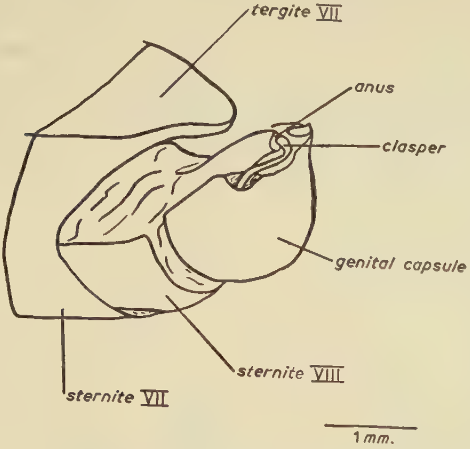
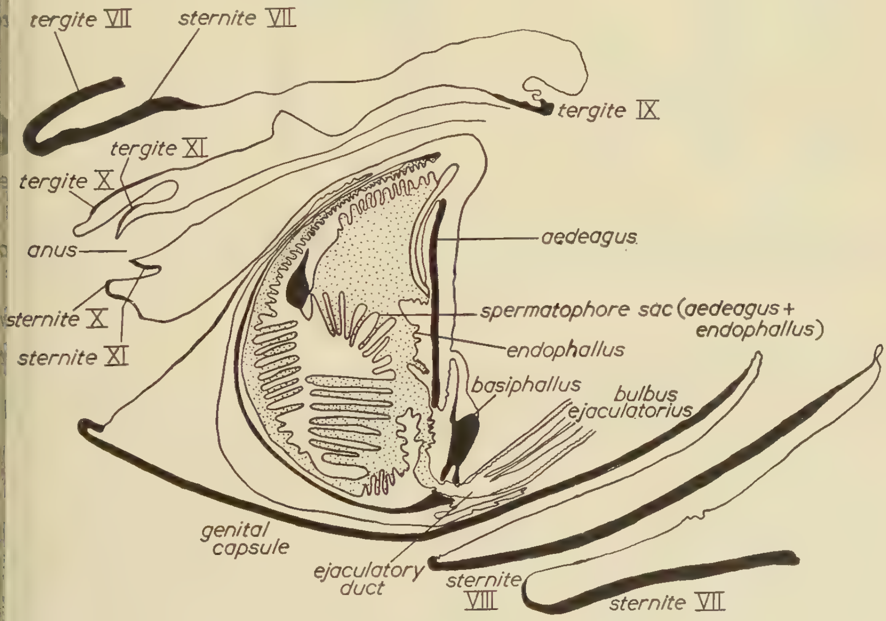


FIG. 2. The terminal segments of an adult male *Rhodnius*: left lateral view with wings removed.



IG. 3. Diagram of a median longitudinal section of the terminal segments of the male *Rhodnius*.

lesignated as the spermatophore sac. Further, it is evident that the wall of the edeagus is made up of two layers of cuticle bounding a blood-space and that he wall of the spermatophore sac is similarly double. It is suggested that only he outer wall of the aedeagus is in fact aedeagus and that the inner wall is the ndophallus. Similarly, the wall of the spermatophore sac is composed of edeagus on the outer side and of endophallus on the inner or lumen side.

A consideration of fig. 4 makes the reasons for this suggestion more clear. Fig. 4, A represents the condition found in many insects—a basiphallus and tubular aedeagus with a membranous endophallus. In fig. 4, B the terminal part of the entire phallic structure has invaginated to form an internal sac and it is suggested that this is what has happened in *Rhodnius*.

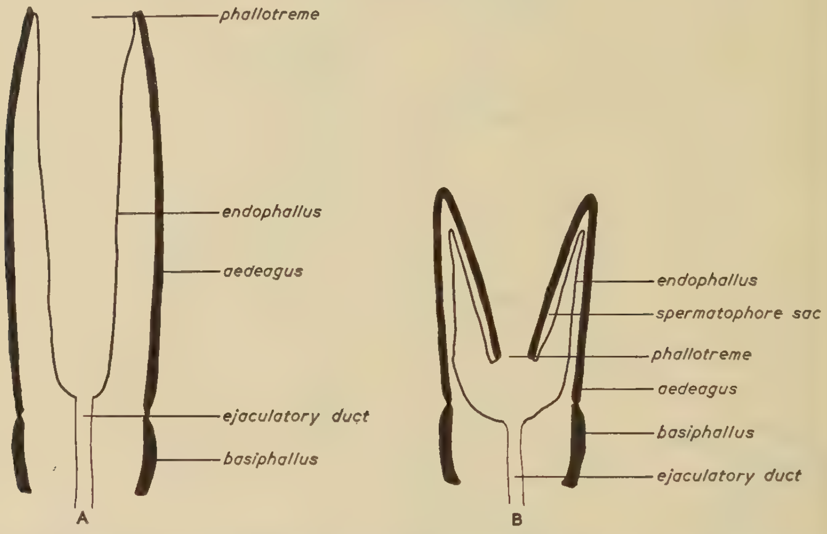


FIG. 4. Diagram illustrating the development of the spermatophore sac from the generalized condition. The primitive arrangement is represented in A (after Snodgrass, 1935). B, achieved by the folding in of the terminal portion of the penis in A, is a diagram of the condition found in *Rhodnius*.

The events at copulation

In order to study the processes occurring at copulation, it was necessary to kill the mating pairs at various times after the initiation of copulation. To this end, pairs of recently fed *Rhodnius* were confined in glass vials in an incubator at 28° C, and the progress of mating observed through the glass front of the incubator at intervals of 3 min. When the desired stage had been reached, the vial was removed from the incubator and boiling aqueous Bouin's fluid was poured over the insects. The pair was then dissected to free the male genitalia from the female. The following description is based on material obtained in this way.

During copulation the male takes up a position beside and slightly ventral to the female. Since the penis is normally directed towards the posterior, this position requires that the genitalia, including the genital capsule, be rotated through almost 180°, as has been described in some detail for *Oncopeltus* (Bonhag and Wick, 1953). Within 3 min of the beginning of copulation, the aedeagus has been extended into the bursa copulatrix of the female (fig. 5, A). Later, at 6 min, the spermatophore sac becomes visible (fig. 5, B), having been everted out of the aedeagus. Eversion of the spermatophore sac continues

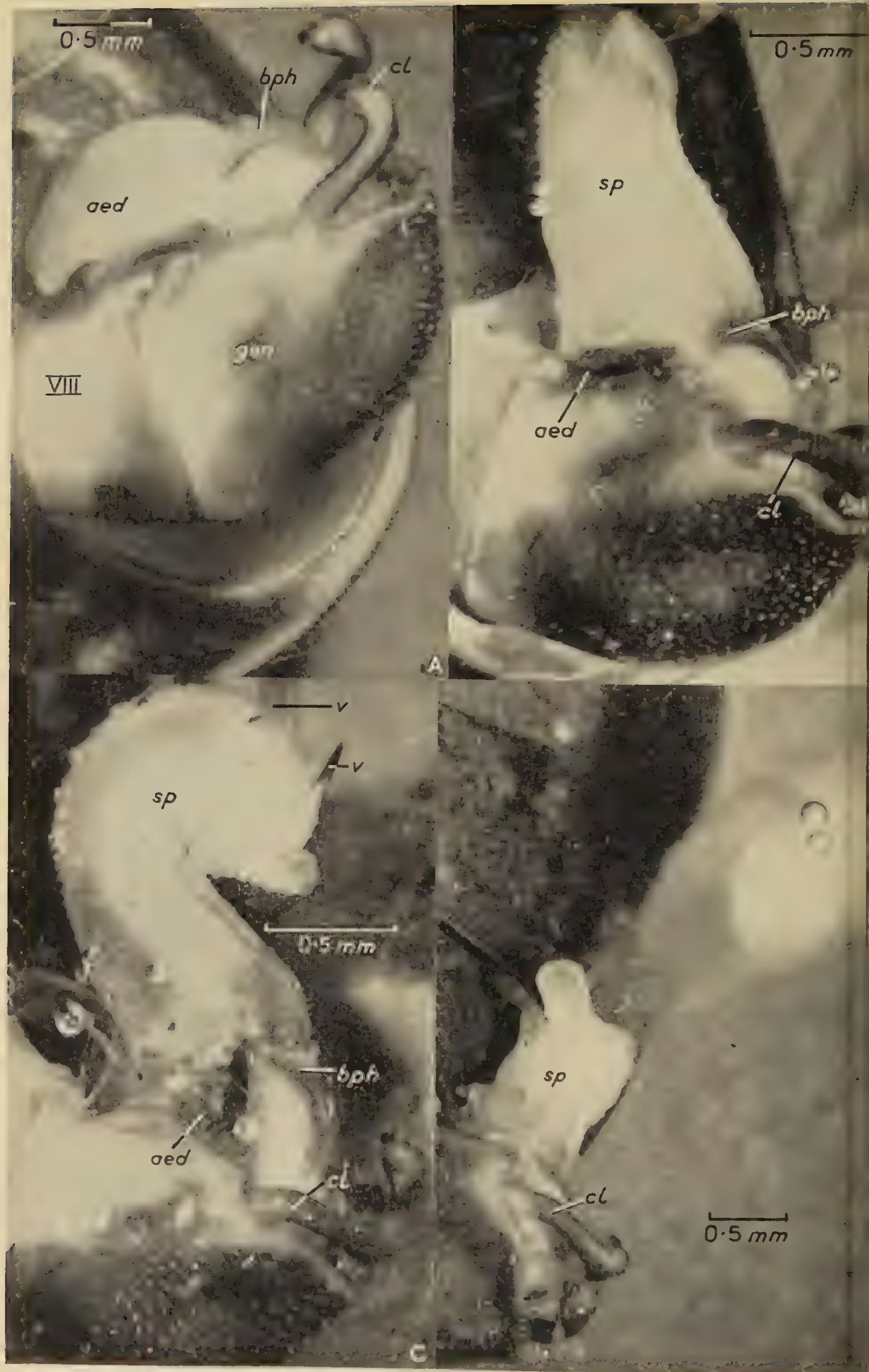


FIG. 5

K. G. DAVEY

ntil, at 10 min after the beginning of copulation, it reaches the fully everted ndition shown in fig. 5, C. Dissection of the spermatophore sac at this stage veals that it contains a fully formed spermatophore. Within about 12 min e spermatophore sac has collapsed after ejecting the spermatophore (fig. 5,). Copulation normally occupies about 30 min; the remainder of the time is

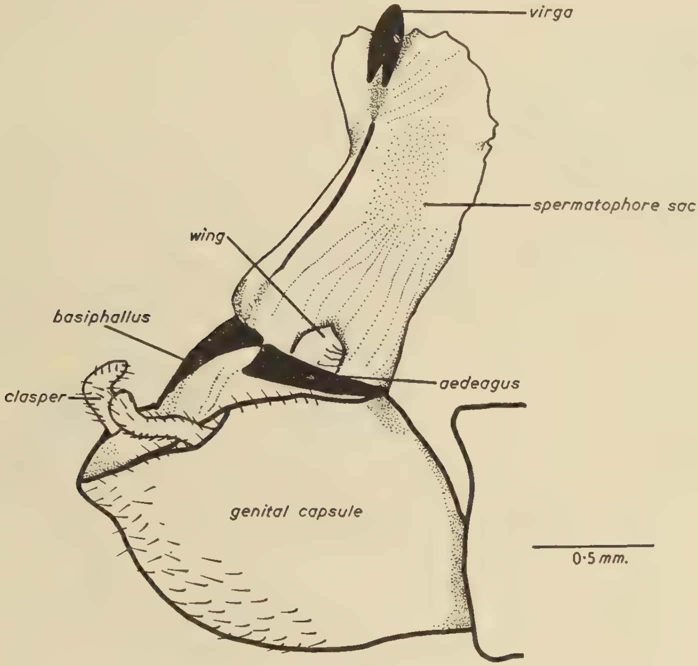


FIG. 6. Drawing of a partially erect penis of *Rhodnius*, left lateral view.

ken up with the return of the spermatophore sac to its normal position side the aedeagus. This process has not been studied.

The intromittent organ is difficult to dislodge from the female when the ermatophore sac is fully everted. This is presumably brought about by two teral extensions of the spermatophore sac at its base, referred to here as ings (fig. 6). Since the distance between the tips of the wings is greater than e diameter of the female gonopore, the male genitalia cannot be withdrawn ntil the spermatophore sac has collapsed. The spermatophore sac also bears

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FIG. 5 (plate). Photographs of the male genitalia of *Rhodnius at* various times after the tiation of mating.

3 min after; only the aedeagus is visible.

6 min after; the spermatophore sac is partially everted.

10 min after; the sac is completely everted.

12 min after; the sac has collapsed and the spermatophore has been ejected.

aed, aedeagus; *bph*, basiphallus; *cl*, clasper; *gen*, genital capsule; *sp*, spermatophore sac; *sph*, spermatophore; *v*, virga; *VIII*, eighth sternite.

three sclerotized plates, or virgae, consisting of a spade-like terminal plate an a pair of longer lateral plates (figs. 5, C; 6).

A median longitudinal section of the everted genitalia reveals certai details (fig. 7). The fully formed spermatophore may be seen within th spermatophore sac and this diagram shows the phallotreme, or termina opening of the spermatophore sac through which the spermatophore i

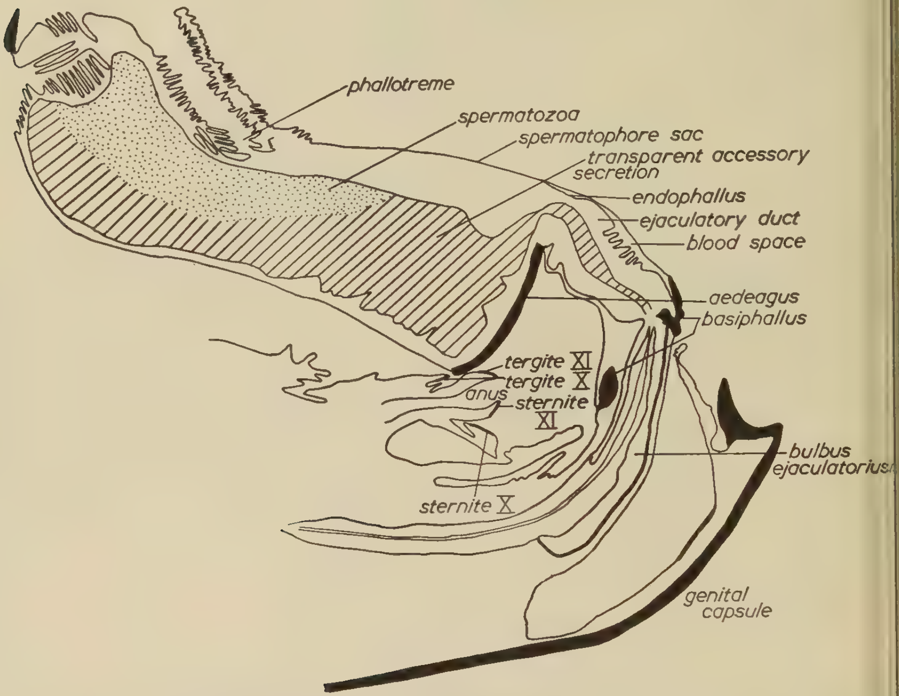


FIG. 7. Diagram of a median longitudinal section of the terminal segments of a male *Rhodniu* with a fully everted spermatophore sac.

finally released. The folds of the spermatophore sac projecting into the lume appear to be responsible for the formation of the slit which contains the semen

Among the stains used for these sections was Mann's methyl blue / eosin According to Pantin (1948), methyl blue takes the properties of a basic stain in the presence of eosin. In the spermatophore sac the material forming th main body of the spermatophore is strongly eosinophil (cross-hatched portio in fig. 7). In the common duct within the bulbus ejaculatorius and in th accessory gland and its duct, however, the material which is continuous wit the spermatophore stains strongly with methyl blue. It is apparent that som change occurs in this material at the level of the intromittent organ.

The transparent accessory glands

The internal organs of reproduction of the male of *Rhodnius* can be seen i fig. 8. They consist on each side of a testis, a vas deferens which swells in its ddle third to form the seminal vesicle, and the accessory glands with their ct, which joins the vas deferens of the corresponding side in the bulbus culatorius. The two bulbi unite in the mid-line to form the ejaculatory duct ening at the base of the penis. The accessory glands are of two types: three the four lobes on each side are filled with a transparent mucilaginous fluid; : fourth contains the opaque secretion referred to earlier.

Khalifa (1950) noted that the transparent glands contain little secretion in

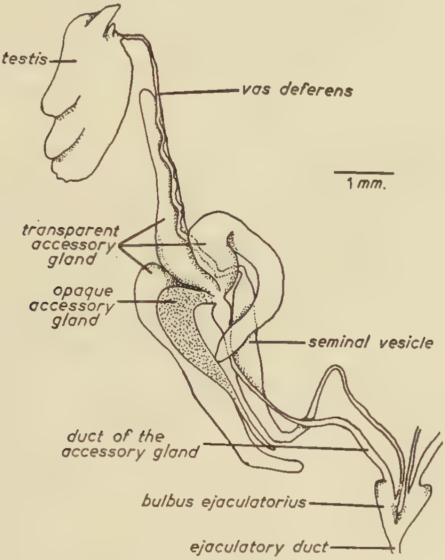


FIG. 8. Drawing of the left side of the male reproductive system of *Rhodnius*, dorsal view.

ırved males, which are unable to produce spermatophores, and he inferred ›m this that the transparent secretion made up the main body of the ermatophore. A more direct demonstration of this was obtained in the esent study by mating normal females to males which had one or more ›es of the transparent accessory glands removed. Such matings resulted in : production of smaller, but otherwise normal spermatophores. Removal of : opaque glands, on the other hand, had no effect on the main body of the ermatophore.

The transparent secretion resembles the material making up the main body the spermatophore in every apparent way except fluidity. Simple histo-emical tests, performed as outlined in Pearse (1953), on whole spermato-ores and on whole and sectioned transparent accessory glands fixed in utral formaldehyde solution revealed a further correspondence in proper-s. Both were strongly positive to Millon's test, demonstrating the presence protein. Fat was absent from both spermatophores and accessory glands, shown by their failure to stain with Sudan black B or Sudan red colouring ents. The presence of polysaccharide in both materials was demonstrated by a very strong periodic acid / Schiff reaction. These results pointed to protein-polysaccharide complex, and additional tests were performed with view to characterizing the substance. Neither exhibited metachromatic staining with toluidine blue, and a methylene blue extinction test performed o spermatophores only gave a methylene blue extinction value which wa indefinite, but certainly greater than 5. According to Pearse (1953), material exhibiting these properties can be designated as mucoproteins or neutr mucopolysaccharides. At any rate, the transparent accessory secretion appear to differ from the material in the spermatophore only in consistency; th spermatophore is simply a more solid transparent secretion.

The importance of pH

A clue to the processes involved in the solidification of the transparen secretion to form the spermatophore was gained from a consideration of th behaviour of the freshly formed spermatophore in various environments. spermatophore removed from a recently mated female swelled slightly i distilled water, and slowly liquefied in more basic solutions, such as tap wate In slightly acid solutions, the spermatophore remains unchanged.

The effect of pH on the consistency of the transparent secretion was studie by placing a single lobe of the gland in insect Ringer which had been buffere to the desired pH with isotonic sodium hydrogen phosphate / potassiun dihydrogen phosphate buffer. After 10 min the gland was dissected and th condition of the contents noted. The pH values employed lay between 5·0 an 6·8 and were separated by 0·2. Above pH 5·6 the material was quite fluid, bu at 5·4 and 5·6 the secretion had gelled to form a material closely resemblin that in the spermatophore. At pH 5·2, the material was again fluid. Thi experiment was repeated twice and a fourth trial was performed with McIlvaine's standard buffer; identical results were obtained in each case. It i clear that the mucoprotein of the accessory gland will solidify at a pH of abou 5·5 to form a material like that in the spermatophore.

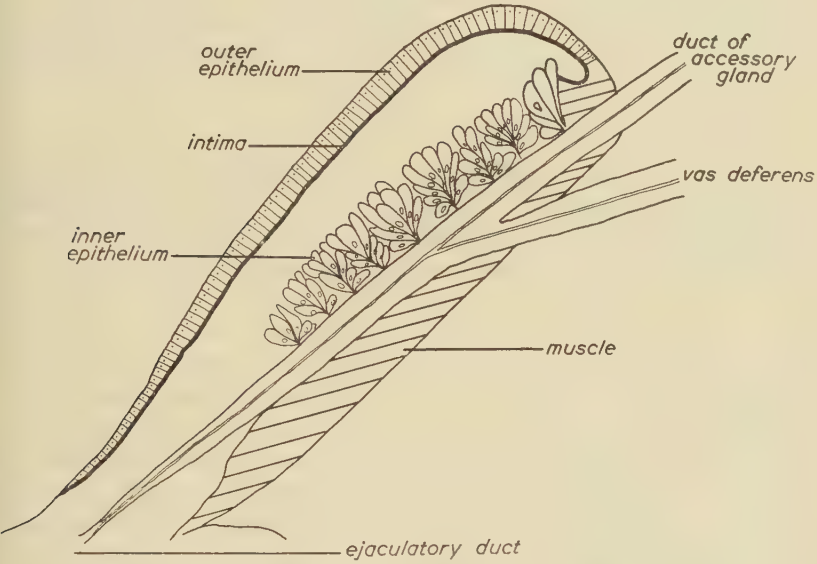
The pH of the reproductive ducts

It is in the male system that the spermatophore material exists as both fluid and a solid, so that the pH of the various ducts is of interest. Since th amount of fluid in these ducts is very small, the organ in question was crushe on to a tiny piece of B.D.H. indicator paper. In this way it was found that th accessory glands, testis, and ducts down to the level of the bulbus ejaculatoriu were at or near neutrality, while the pH of the fluid in the lumen of the bulbu was about 5·5. Only the bursa copulatrix of the female was examined; it als was at pH 5·5.

In order to confirm these observations, certain properties of methyl re were employed. In addition to its well-known action as a pH indicator with a end point near 5·5, this dye can also be used as a vital stain. A 1% solutio in 0·9% NaCl was injected into the haemocoele of 10 males, which wer dissected in 0·9% NaCl 5 h after injection. The ducts and organs of th productive system of such males were stained yellow, indicating a pH nsiderably above the end point, whereas the fluid in the bulbus was stained ange, indicating the end point pH of 5·5. To confirm the pH of the bursa the female, a small crystal of methyl red was inserted into the bursa through e genital opening. After 5 h the bursa was stained with the end-point colour.

he bulbus ejaculatorius

It appears from the foregoing that the lowering of pH at the level of the ılbus may be responsible for the solidification of the transparent accessory



G. 9. Diagram of a median longitudinal section of the bulbus ejaculatorius of the male *Rhodnius*.

cretion to form the spermatophore. In order to test the ability of the bulbus clot the transparent secretion, the bulbi from 20 males were macerated with rceps in 1 ml of insect Ringer previously adjusted to pH 7 with 0·2 N NaOH. ts of the vas deferens and accessory duct from the same animals were acerated in another sample of the same Ringer. A lobe of the transparent cessory gland was placed in each suspension. After 10 min the secretion in e Ringer containing the bulbi had solidified, while the other remained fluid. oreover, the pH of the Ringer containing the bulbi had fallen to 5·5, while at of the other suspension remained near neutrality. Because of the large ımbers of insects required, this experiment could be repeated only once; on at occasion similar results were obtained. Other experiments using substantially fewer insects were unsuccessful.

The anatomy of the bulbus (fig. 9) is so arranged that the products of the cessory glands are kept separate from the secretion of the bulbus up to the vel of the penis. The outer wall of the bulbus is a single layer of epithelium lined with an intima, except on the medial side, where the wall is formed b muscle within which the combined accessory and sperm ducts run. Projectin from this common duct into the lumen is the inner epithelium, which presum ably secretes the greenish, watery fluid that fills the lumen of the bulbus. Thi fluid was not fixed by any of the fixatives used, and, as we have already see has a pH of 5·5. The structure is, of course, repeated on the other side of th animal and the outer epithelial layers from each bulbus unite to form the shor ejaculatory duct opening into the base of the penis. The common ducts from each side empty separately into the ejaculatory duct. Thus the fluid secretio from the transparent glands is kept separate from the acid secretion of th bulbus until they are allowed to mix in the penis. It is worth rememberin that this is the region in which the transparent secretion becomes eosinophi in Mann's stain.

CONCLUSION

On the basis of these observations it is concluded that the spermatophor of *Rhodnius* is composed of a mucoprotein or neutral mucopolysacchari originating in the transparent accessory glands of the male and that this secretion solidifies in the spermatophore sac of the male intromittent organ as result of a change in pH from near neutrality to 5·5. This change in pH i brought about by the secretion of the bulbus ejaculatorius of the male. Th entry of the transparent secretion into the spermatophore sac causes th eversion of that organ, which acts as a mould for the spermatophore.

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A Study of Rawitz's 'Inversion Staining'

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SUMMARY

The Rawitz method involves mordanting with tannic acid and potassium antimony trate, and staining with basic fuchsine. The mordanting causes basic fuchsine to t as though it were an acid dye ('inversion staining').

A modification of the method is described in the present paper. This modification kes it possible to obtain the same results in a shorter time.

The chief substances stained by Rawitz's method are phospholipids, certain pro- ns, and certain polysaccharides.

Although the method cannot be regarded as a cytochemical test in the strict sense, t it gives useful indications of chemical composition and in addition is valuable to the rphological cytologist as a technique for showing certain cytoplasmic inclusions itotic spindle, acrosome, mitochondria, 'Golgi apparatus' of certain cells).

INTRODUCTION

T is well known that the so-called 'Golgi apparatus' is extremely difficult to reveal by any staining method. Baker, in the course of his investigation on s organelle in the epididymis of the mouse, found that it can be stained by sic fuchsin after a special mordanting process (1957). The method was taken m Rawitz (1895), who found that basic fuchsin, if mordanted with tannic d and potassium antimony tartrate, loses the character of a dye for chro- tin and colours the cytoplasm instead. Rawitz called this effect 'inversion ining'. Since this technique, when applied to various kinds of cytological terial, gave good selectivity in visualizing certain delicate cell structures, it med interesting to investigate the nature of the chemical compounds which responsible for positive Rawitz staining.

THE METHOD OF 'INVERSION STAINING'

The staining procedure as devised by Rawitz himself consists of the follow- steps. Paraffin sections ($5\ \mu$ thick) of tissue fixed in strong Flemming's id, prepared in the usual way, are mordanted in a 20% aqueous solution of nic acid for 24 h. The sections are then rinsed with a 2% aqueous solution potassium antimony tartrate (tartar emetic) and left in a bath of the same ution at 37° C for $2\frac{1}{2}$ h. They are then stained with basic fuchsin (saturated oholic stock solution, diluted with distilled water 1 : 1) for 24 h and differ- iated with 2·5% tartar emetic solution for a further 24 h. They are then ydrated and mounted in Canada balsam in the usual way. The Rawitz thod leaves the nuclei unstained, but shows such organelles as mitochondria the Golgi complex of certain cells in brilliant pink.

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In the present investigation a number of experiments on mouse epididymi were done in order to elucidate the importance of each step in the procedur It has been shown that the mordanting with tannic acid followed by tartrat emetic treatment is essential for the inversion method; and if the preparatio was not previously treated by strong tannic acid and potassium antimony tar trate, according to the schedule given, the differentiation with dilute tannic aci after basic fuchsin staining had no visible effect at all. The fixative used is als of the first importance. Of four different mixtures used in my experiments— Flemming, Bouin (with and without postchroming), Helly, and Hermann— Flemming gave the best results. The influence of further handling of th tissue, such as the duration of washing after Flemming fixation and of dehy dration, was also investigated. It has been found that variation in periods o washing (5 or 24 h) and of treatment with 70% alcohol, even up to 15 h, has n effect upon the picture obtained. As the whole procedure takes 6 days som experiments were undertaken with the intention of shortening it, if possibl The period of mordanting, which was based on long experience of the dy industry (Rawitz, 1894) was kept unchanged. The duration of two furthe steps, staining and differentiation, was examined carefully. It was found tha the period of staining with basic fuchsin may be shortened considerabl However, a shortening of the period of differentiation and the use of a highe temperature (37° C instead of room temperature) did not give good result A higher temperature causes darkening of the tissue, and turns the colou from pink to violet, which results in a picture of poorer contrast. After man different trials it was found that the best time for the staining was 3 h, and fo differentiation 18 to 20 h.

The old and the new procedures are compared in table 1.

The use of Baker and Jordan's adhesive albumin (1953) instead of Meyer adhesive mixture gives preparations which dry rapidly and are ready fo further handling in 1 or 2 h.

The two techniques give the same results. After the modified procedur both the mitochondria and the Golgi complex of certain cells can be visualize In all further work I used the shortened procedure only.

ANALYSIS OF THE TECHNIQUE

Investigation of animal tissues: epididymis and pancreas of mouse

A very striking fact in the Rawitz test is that chromatin remains unstaine after applying a basic dye. The question arose whether there was a lack DNA in the nuclei, possibly through extraction by mordanting. Howeve a positive Feulgen reaction after Rawitz mordanting proved that this was n so. It is evidently not a lack of DNA in the nuclei that causes them to remai unstained. Both DNA and RNA remain unstained after this method. I hav been able to prove this by the application of the whole Rawitz procedure t pancreas cells previously treated by RNA-ase (according to the method give by Bradbury, 1956). No diminution in the intensity of staining was caused b atment with RNA-ase. It follows that RNA is not responsible for Rawitz ining. A suggestion as to the cause of the reaction arose from histochemical estigations on lipids carried out by Dr J. R. Baker and several workers in laboratory. Clayton obtained a selective positive Rawitz staining of the osome in late spermatide of the house cricket (1957). It is known that this ganelle contains phospholipids (Clayton, Deutsch, and Jordan-Luke, 1958). adbury applied the test to leech adipose cells. Structures described in his

TABLE 1

A comparison of the new and old procedures in 'inversion staining'

	Original Rawitz technique	*Modified technique*
day	9 a.m. Fixation in strong Flemming's fluid	as in procedure I
d day	9 a.m.–2 p.m. Washing in tap-water	,,
	2–3.30 p.m. Dehydration in grades of alcohols	
	3.30–5.30 p.m. Paraffin embedding	
d day	9 a.m. Cutting of sections	,,
	12 midday. Mordanting in 20% tannic acid (24 h)	
h day	12 midday–2.30 p.m. A short rinse in tartar emetic (a few seconds), followed by tartar emetic bath at 37° C	,,
	2.30–2.35 p.m. Tap-water and rinsing in distilled water	
	2.35 p.m. Staining in basic fuchsin (24 h)	2.35–5.35 p.m. Basic fuchsin staining
		5·35–5·45 p.m. Tap-water and rinse in distilled water
		5.45 p.m. Differentiation for about 18 h
h day	2.30–2.45 p.m. Tap-water and rinse in distilled water	12 midday. Rinse in water, dehydration, mounting in Canada balsam
	2.45 p.m. Differentiation with dilute tannic acid for 24 h	
h day	11 a.m. Rinse in water, dehydration, mounting in Canada balsam	

evious investigation as giving a strong acid haematein reaction (1956) were o selectively stained by the Rawitz method (1957). The test applied by me mouse pancreas showed a positive staining of ergastoplasm, which accord- to Clayton and Bradbury contains phospholipids (1958). These data ggested that phospholipids, among other compounds, may be responsible the Rawitz reaction.

vestigation of the reaction with pure substances

To obtain more accurate data about the substances responsible for Rawitz ining, investigations were made on a number of pure substances and on me compounds isolated from animal tissues. The Rawitz reaction was plied to over 120 different compounds which are representative of various substances found in the cell (compare Baker's study of the acid haematein te (1946)). Different solvents, according to the nature of the investigated compounds, were used to prepare strong solutions (about 10%), which were the forced into elder-pith cubes (of side 1 mm), according to the method given b Kaufmann and Lehmann (1926). The cubes, saturated with the various compounds, were handled in exact accordance with the procedure established fo inversion staining. The phospholipid fractions used in experiments we isolated from calf brain according to the methods given by Desnuelle (195 and by Peach and Tracey (1955). Carotene was extracted from carrot root Several experiments were done with native proteins and also with modifie proteins. Acetylated, desaminated, and methylated albumin, and guanidylated casein were prepared according to the methods cited by Greenber (1951). In addition a few substances were investigated in their natural pos tions in tissue sections (collagen, fibroin, chitin, lignin, starch, and cellulose

The results are summarized in the Appendix (p. 237).

A negative reaction (absence of colour) may be caused either by lack o affinity of the substance investigated for the reagents, or by the fact that th substance was washed out from the pith cells by reagents used during th procedure.

Only the following compounds were Rawitz-positive: lecithin, cephalin sphingomyelin, carotene, cellulose, lignin, starch, dextrine, glycogen, gelatin collagen, fibroin, esterified serum albumin, guanidylated casein, mucin (th mucoprotein of goblet cells).

DISCUSSION

It is difficult to interpret the results presented above. All three main steps o the procedure described—fixation, mordanting, and staining—appeared to b essential for the inversion effect. The influence of the fixation mixtures is of double nature. Each component of the fixative mixture (that is, each simpl fixative, in Baker's sense (1958)), by binding the various chemical constituent can make them insoluble and thus prevent their diffusion from the tissu Alternatively, by attacking some of the chemical groups, fixatives can split th more complex compounds and thus make their components accessible to othe reagents. It seems that in the case of Flemming's mixture both possibilities tak place. Recent investigation by Bradbury and Clayton confirm the ability o Flemming's mixture to unmask certain chemical constituents of the cytoplasm (1958). In the present investigation we have shown that no inversion effec can be obtained after Bouin's fluid. Of the four fixative mixtures used, onl those containing OsO_4 and CrO_3 gave positive results. Rawitz himself mentioned that when he used fixatives other than Flemming's, he obtained onl diffuse staining. According to him the presence of strong chromic acid is o great importance (1895). The fixation of tissue by OsO_4 and CrO_3 seems t facilitate the action of the mordant on the different cell components. Th mordant used by Rawitz was tannic acid followed by potassium antimon tartrate. Rawitz was guided to this method by the experience of the dye in stry. The basic anilin dyes, used without any previous treatment of the re, have an affinity for wool and silk, but not for cotton. After tannin/tartrate rdanting they are able to attach themselves to cotton. The 'inversion effect' due to the reaction between the cell constituents and tannic acid. As tanning s been used for many centuries in industrial practice for the preparation of ather and in cotton dyeing, great attention has been paid to explaining this npirical procedure. Nevertheless it is still far from being completely under- od. The results of my investigations indicate that of the various chemical mpounds present in cells, only a small number can be considered as tannin- sitive. These compounds belong to different chemical groups. The most nportant groups of compounds shown to be Rawitz-positive are phospho- ids, certain proteins, and certain polysaccharides. It is supposed that the action between tannic acid and collagen consists in the formation of links tween the OH groups of the tannins and the amino or carboxyl groups of e protein (Thorpe and Whiteley, 1937–54). On the basis of our results we n say little about the nature of this tannic acid / protein reaction. Our ex- riments show that albumin and casein, which are Rawitz-negative, become awitz-positive after transformation into a strongly basic form (as for example ter guanidylation or estrification). The same native proteins, on changing eir nature into a more acidic one (by acetylation and desamination) retain eir Rawitz-negative character. This suggests that the ability of tannic acid react with proteins depends—among other things—on the basic character the proteins. The slightly positive staining I obtained in spermatozoa in the ididymis of the mouse seems to confirm this suggestion, since they are rich basic protein. However, the negative result obtained with purified pro- mine sulphate and clupein indicates that the reaction with tannic acid must more complicated than the other results would suggest.

It follows from my investigation that the second group of chemical con- ituents which are influenced by mordanting with tannin is phospholipids. delberg (1953), in his investigations of the action of tannic acid on the ythrocyte membrane, obtained some data which are in agreement with ours. sing various artificial films as models of the cell membrane, he found that nnic acid can react with films of lecithin and cephalin, whereas no reaction as observed with oleic acid or cholesterol. It is possible that the phosphate roup in the phospholipid molecule is responsible for the reaction with tannic id.

The reaction of tannic acid with highly polymerized carbohydrates seems ost difficult to interpret. It is known that tannic acid can decompose carbo- ydrates (Knecht and others, 1916) but the nature of the reaction is still not ear. The positive Rawitz reaction shown by mucin in the goblet cells of the ouse ileum may be connected with this process.

It is well known that tannins have the ability to form compounds with basic yes. These compounds are not very fast. They are insoluble in water but are luble in excess of tannic acid. To obtain better fastness the tannic acid is rst converted, by means of metals or metalloids, into insoluble compounds which have the same ability to form compounds with basic dyes. Of the various metals used for this purpose the best fixing agent is antimony (Knecht and others, 1916). Exactly the same procedure is applied in the Rawitz inversion test. The complexes of tannic acid with certain cellular compounds (proteins, phospholipids, or carbohydrates) are converted into insoluble compounds by means of antimony tartrate, and they are visualized afterwards by means of basic fuchsin as fast, coloured compounds. The last step of the reaction—differentiation with dilute tannic acid—is applied in order to remove the dye which has been taken up directly and which has not reacted with any tannate / antimony complex. Some of the tannin / basic fuchsin complexes are probably dissolved away at the same time. Some of the remaining dye is certainly removed during the process of dehydration of sections. Thus the final picture obtained, provided that all the steps of the method were carefully performed, shows the places in the cell where either phospholipids, or carotene, or highly polymerized carbohydrates, or certain tannophil proteins are present.

THE RAWITZ METHOD IN THE INVESTIGATION OF CELLULAR ORGANELLES

Baker, who re-introduced the Rawitz method, used it to reveal the Dalton complex (the so-called 'Golgi apparatus') in the epididymis of the mouse. The picture was exactly the same as obtained after the classical osmium methods. In the course of our investigations we also obtained a very distinct staining of mitochondria in the ileum of the mouse. Rawitz himself used the method in the investigation of the testis of urodeles. Resting cells exhibited a positive staining of the centrosome and attraction-sphere (idiozome). In mitotic nuclei only the spindle was stained selectively (1895, 1895*a*). The presence of tannophil cell-components was noted by Salazar and his co-workers. In his papers on the subject, published since 1920, he reported the presence of the 'tannophil chondriome' (1921), 'tannophil elements in the Golgi zone' (1932), and tannophil mucigens in intestinal cells (1926). Salazar detected the tannin bound to these elements by means of the dark blue colour of its iron salts. The test was performed after the use of different fixatives, preferably Bouin's fluid (1920). In certain experiments Salazar introduced osmium tetroxide to the method as a new agent after tannin mordanting ('accentuation osmique', 1921). No precise tests for establishing the chemical compounds responsible for the reaction were given. The Rawitz technique seems to stand on a different and better-established basis.

All the facts mentioned above, taken in conjunction with the results of our analysis of the technique, seem to indicate that Rawitz's inversion staining can be applied as a useful cytological method. For the morphologist it is a good method of showing certain delicate cellular organelles that are difficult to reveal in any other way. The method can be useful also to the cytochemist. To use Panijel's term (1951), it gives a 'reaction signalétique'; that is to say, it provides information as to which cytochemical tests are likely to give instructive information.

I should like to express my gratitude to Dr. J. R. Baker for suggesting and

pervising this investigation and for many stimulating and helpful discus-
ons; also to Sir Alister Hardy, F.R.S., in whose Department the work was
rried out.

My best thanks are also due to all the members of the Cytological Labora-
ry, Department of Zoology, Oxford, and to Dr. H. Dominas and Mgr. J.
arolczyk of the Nencki Institute in Warsaw for their friendly help.

APPENDIX

Results of applying the Rawitz technique (modified) to known substances

Groups of substances	*Rawitz-negative (no colour)*	*Rawitz-positive (pink)*
raffins	medical paraffin	
	paraffin m.p. 56	
tty acids	*n*-butyric acid	
	palmitic acid	
	stearic acid	
	oleic acid	
	ricinoleic acid	
	fatty acids—mixture extracted from larvae of *Galleria mellonella*	
	myristic acid	
aps	sodium palmitate	
	,, stearate	
	,, oleate	
	potassium oleate	
	,, laurate	
	,, butyrate	
	calcium oleate	
thyl esters of fatty acids	ethyl stearate	
	ethyl palmitate	
	ethyl oleate	
	ethyl laurate	
riglycerides and mono-glycerides	tributyrin	
	tristearin	
	glyceryl monostearate	
hospholipids	cerebrosides	egg lecithin
		brain lecithin (crystallized)
		brain lecithin+cephalin (mixture)
		brain cephalin (crystallized)
		brain sphingomyelin (crystallized)
		brain phospholipids (mixture, crystallized)
atty waxes	carnauba wax	
	beeswax (white)	
	spermaceti	

Groups of substances	*Rawitz-negative* (*no colour*)	*Rawitz-positive* (*pink*)
Sterols and their esters	cholesterol	
	cholesteryl stearate	
	cholesteryl oleate	
	lanoline	
	ergosterol	
Carotenes		carotene
Organic acids other than fatty acids	benzoic acid	
	citric acid	
	oxalic acid	
	tannic acid	
Aliphatic alcohols and their derivatives	glycerol	
	sodium β-glycerophosphate	
	sorbitol	
Phenols and their derivatives	phenol	
	cresol	
	adrenalin hydrochloride	
Essential oils and oleoresins and their constituents	pinene	
	eugenol	
	vanillin	
	origanum oil	
	clove oil	
	cedarwood oil	
	Canada balsam	
	terpineol	
Carbohydrates and related substances	dextrose	lignine (in stem of *Tradescantia viridis*)
	lactose	
	sucrose	dextrine
	xylose d(+)	starch (soluble)
	maltose	starch in potato tubers
	chitin	glycogen
		glycogen in rabbit's liver
Amino-acids, proteins, enzymes, products of protein metabolism	L-tyrosine	
	cysteine hydrochloride	
	egg albumin	esterified serum albumin
	acetylated s. albumin	
	miogen	
	aktyn	
	fibrinogen	
	casein	guanidylated casein
	pepsine	
	protamine sulphate (salmine sulphate from salmon milt)	
	clupein	
	globin from hemoglobin	collagen (in mouse ileum)
		gelatin
		fibroin (in silk glands of *Bombyx mori*)
	trypsin	
	nucleoprotein	
	urea	
Purine derivatives and their compounds	guanine	
	uric acid	
	nucleic acid (nucleinic acid from yeast)	

Groups of substances	*Rawitz-negative (no colour)*	*Rawitz-positive (pink)*
nclassified	nucleic acid (Na salt) ribonucleic acid in mouse pancreas (ergastoplasm) DNA—in nuclei of mouse tissues chlorophyll (BDH) chlorophyll (plant chloroplasts) nicotine	

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The Feulgen Reaction in the Different Stages of the Life-cycles of Certain Sporozoa

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SUMMARY

The nuclei of the male and female gametocytes of the malarial parasites (*Plasmo- m* and *Hepatocystis*) are Feulgen-negative, while the nuclei of the gametocytes of *patozoon*, which are not sexually differentiated, are Feulgen-positive. The nuclei the male gametocytes of *Eimeria* are strongly Feulgen-positive, while the nuclei of female gametocytes are Feulgen-negative. The nuclei of the male gametes of *asmodium* are Feulgen-negative, while the nuclei of the male gametes of *Eimeria* are ongly Feulgen-positive. DNA appeared to be present in the gametocytes of *Plas- dium* when the fluorescence method of Armstrong (1956) was employed.

Feulgen-positive inclusions have been found to occur in the cytoplasm of some of e oöcysts of *Plasmodium*. The oöcysts containing such inclusions may degenerate ally or partially. There is no evidence to relate this type of degeneration with the mation of Ross's black spores. These inclusions have been found in abundance in oöcysts of *P. cynomolgi*, while in those of *P. gallinaceum* they were found only once.

Certain Feulgen-positive granules have been found in the cytoplasm of some of the cysts and the female gametocytes of *Eimeria*. These granules did not appear to be ated to any degenerating changes. A probable loss of some of the nuclei during orogony of *Eimeria* is discussed.

The nuclei in the early pre-erythrocytic schizonts of *Plasmodium cynomolgi* are ulgen-positive, notwithstanding an earlier report to the contrary.

INTRODUCTION

THE nucleus of the various species of malarial parasites has long been an object of interest. Attempts have been made from time to time to stain is nucleus by the Feulgen reaction. Most of the earlier studies (Pawan, 31; Jírovec and Černý, 1932; Breindl and Jírovec, 1932; Missiroli and osna, 1934; Ungo Mugdan, 1938; Chen, 1944; Deane, 1945; Schäffer, 45; Lillie, 1947; Lewert, 1952; and Sen Gupta and others, 1955) conrned the erythrocytic stages of the parasite only. In these earlier studies me of the authors reported a negative reaction in some or all of the erythrotic stages, while other authors reported a positive result of varying degree intensity. One of the authors (Deane, 1945) reported that the elimination the malarial pigment resulted in an improvement of the Feulgen reaction the nuclei of the malarial parasite. In contrast to the erythrocytic stages, sporogony stages of the malarial parasite received scant attention. The ulgen reaction in some of the stages during sporogony was recorded in brief Breindl and Jírovec (1932) and by Missiroli and Mosna (1934), while wert (1952), who examined only the sporozoites of one species, reported egative result. Brief mention of the Feulgen reaction in the tissue stages of

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various species of malarial parasite was made by Ungo Mugdan (1938), Lewert (1952), Jeffery and others (1952), Bray and Garnham (1953), Garnham and Heisch (1953), Garnham and others (1955), and Bray (1957 *a*, *b*, *c*). A curious feature of these accounts of the tissue stages is the reported negative reaction in *Plasmodium cynomolgi* (Bray, 1957*c*) in contrast to the positive reaction obtained in all other species of malarial parasites.

A review of all these earlier works shows that the Feulgen reaction in all the stages of the life-cycle of a malarial parasite has never been carried out before. Besides, certain anomalies and contradictions found in the earlier reports necessitate a thorough reinvestigation. Hence, in the present study the Feulgen reaction has been carried out with three species of malarial parasite (*P. gallinaceum*, *P. cynomolgi*, and *Hepatocystis kochi*) in the different stages of their life-cycles, and the results obtained have been compared with the outcome of similar studies made on some other Sporozoa (*Theileria parva*, *Hepatozoon sciuri*, *H. balfouri*, *H.* sp., *Lankesterella* sp., *Pirhaemocyton* sp., and *Eimeria stiedae*) in their available stages. A previous account of the Feulgen reaction in *Hepatozoon* was that by Garnham (1954) on *H. argantis*, wherein it was reported that the zygote nucleus was Feulgen-negative, while the nuclei during the sporogony stages were Feulgen-positive. A similar observation was also reported in *H. sciuri* (Dasgupta and Meedeniya, 1958). It may also be mentioned that the Feulgen reaction was carried out earlier in various species of *Eimeria* (Sassuchin, 1933, 1935; Cheissin, 1940, 1958; Lillie, 1947; Ray and Gill, 1955; and Patillo and Becker, 1955), and in a species of *Isospora* (Bray, 1954). In these studies on *Eimeria* and *Isospora* the attention was mainly focused on the schizogony and the gametogony stages, while the sporogony stages received little or no attention. The brief account of the Feulgen reaction in the sporogony stages of *Eimeria* given by Patillo and Becker (1955) differs substantially from the observations recorded herein.

The methyl green staining reaction and the fluorescence method for the detection of DNA have also been employed to examine the validity of the results obtained with the Feulgen reaction.

MATERIAL AND METHODS

Plasmodium gallinaceum was studied in the laboratory-bred mosquitoes (*Aedes aegypti* var. *queenslandensis*) and in the experimentally-infected one-week-old chicks (*Gallus domesticus*). The erythrocytic stages and male gametes were studied in blood-smears.

Plasmodium cynomolgi was studied in laboratory-bred mosquitoes (*Anopheles maculipenis* var. *atroparvous*) and in experimentally-infected monkeys (*Macacus rhesus*). The sporogony stages were obtained in smears and in sections of the stomach and salivary glands of infected mosquitoes. The pre-erythrocytic schizonts were studied in sections of pieces of liver of infected monkeys obtained by biopsy. The erythrocytic stages were studied in blood-smears.

The exo-erythrocytic stages of *Hepatocystis kochi* were studied in sections of pieces of liver of naturally-infected monkeys (*Cercopithecus aethiops*) and the gametocytes were studied in blood-smears from the same species of monkey.

The stages of *Theileria parva* known as 'Koch's blue bodies' were studied in sections of pieces of spleen and of the lymphatic glands of the vertebrate host (*Bos taurus*).

The gametocytes of *Hepatozoon sciuri* were studied in blood-smears obtained from naturally-infected squirrels (*Sciurus carolinensis*).

The schizogony stages of *Hepatozoon balfouri* were studied in sections of pieces of liver from naturally-infected jerboas (*Jaculus jaculus*), and the gametocytes were studied in blood-smears from the same animal.

The gametocytes of *Hepatozoon* sp., the sporozoites of *Lankesterella* sp., and the stages of *Pirhaemocyton* sp. were obtained in blood-smears from the frog (*Rana occipitalis*).

The sporogony stages of *Eimeria stiedae* were studied by breaking the hard wall of the oöcysts obtained from experimentally-infected rabbits (*Lepus cuniculus*), by pressure. The tissue stages were studied in sections of the livers of the same rabbits, obtained at the 12th, 13th, 14th, and 20th days of infection.

Blood-smears were fixed in all cases in 80% ethyl alcohol. All other materials mentioned above were fixed in Carnoy's fixative. Trial with a large number of fixatives was out of the question owing to the rarity of most of the experimental material.

The Schiff reagent for the Feulgen reaction was prepared according to the method given by Coleman (1938). The period of acid hydrolysis was 5 to 6 min in the case of the blood-smears, 8 to 10 min for all other tissues. After acid hydrolysis the material was treated with the Schiff reagent for 1½ h in all cases. Unhydrolysed controls were used to verify the true nature of the Feulgen reaction. A 0·5% alcoholic solution of light green was used in most cases as counterstain. A 1% aqueous solution of alcian blue was also used for this purpose. The methods for the removal of malarial pigment, as given by Deane (1945) and Lewert (1952) for obtaining a better Feulgen reaction, were tried, but did not yield the desired result in the species studied here.

The positive Feulgen reaction was compared in all cases with the methyl green staining reaction in the Unna-Pappenheim staining solution.

DNA was detected in some stages of the parasites by the induced fluorescence method (Armstrong, 1956). Acetate buffer (pH 4·3–4·8) was employed. Acridine orange was used in a concentration of 1 in 2,000.

OBSERVATIONS

The following is a brief account of the results obtained in the various stages of Sporozoa.

Plasmodium gallinaceum (fig. 1). The nucleus in some oökinetes was

Feulgen-negative, while in some others the nucleus was faintly Feulgen positive. In the faintly Feulgen-positive nucleus the reacting material wa either evenly distributed throughout the nuclear matrix, or appeared as minute dots.

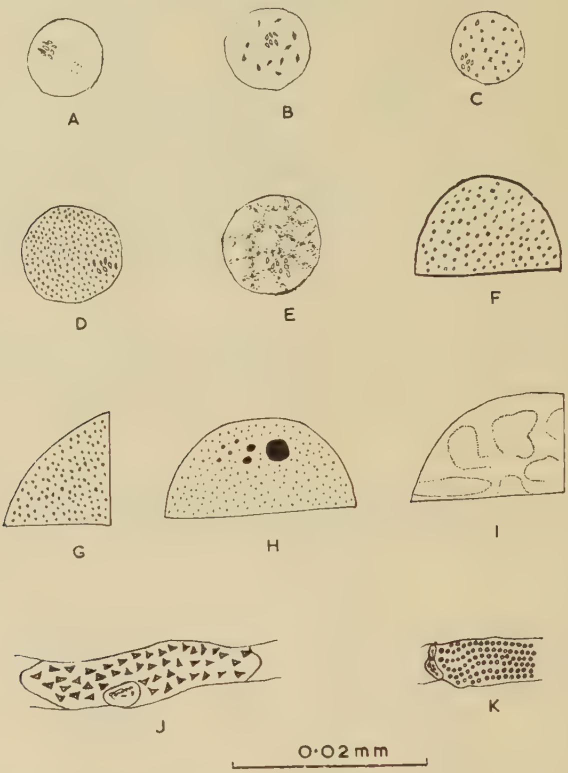


FIG. 1. *Plasmodium gallinaceum.* A, oöcyst 3 days old. A faint and diffuse Feulgen-positive reaction in the single nucleus. The malarial pigments are shown on the left side. B, multinucleate oöcyst 3 days old. Strong Feulgen-positive reaction in the nuclei. C, the same. D, oöcyst 4 days old. Strong Feulgen-positive reaction in the nuclei. E, Feulgen-positive nuclei occurring in the vacuolated cytoplasm of a 4th-day oöcyst. F, Feulgen-positive nuclei in 5th-day oöcyst. G, Feulgen-positive nuclei in a 6th-day oöcyst. H, oöcyst 8 days old. Feulgen-positive inclusions occurring alongside Feulgen-positive nuclei. I, oöcyst 8 days old. Feulgen-positive nuclei of the sporozoites are seen in a mature oöcyst. J, Feulgen-positive nuclei of the exo-erythrocytic schizont. K, Feulgen-positive exo-erythrocytic merozoites.

The resting nucleus of the early uninucleate oöcyst was faintly Feulgen-positive. The reacting material was evenly distributed throughout the nuclear matrix in most of these oöcysts. In some, however, the reacting material was confined to the peripheral region of the nucleus.

The nuclei remained very faintly Feulgen-positive in the majority of the oöcysts up to the 5th day of infection. With methyl green these nuclei were difficult to distinguish. A distinct Feulgen-positive reaction was obtained in minority of the oöcysts even from the 3rd day of infection. The nuclei in ese oöcysts could be easily seen with methyl green.

The majority of the oöcysts after the 5th day of infection were found to ossess strongly Feulgen-positive nuclei. Some of the 5th-day oöcysts showed wo types of nuclei, some strongly Feulgen-positive, others weakly Feulgen-ositive.

In a single oöcyst (8-day-old) large accumulations of Feulgen-positive aterial were noticed in the cytoplasm, in addition to the normal Feulgen-ositive nuclei. These inclusions could not be reconciled with any known ructure of the oöcyst at this stage.

The nuclei of the sporozoites in the mature oöcysts were strongly Feulgen-ositive. These also stained with methyl green. In the majority of the sporo-oites in the salivary glands the nucleus was strongly Feulgen-positive. In ome sporozoites in the salivary glands the nucleus was weakly Feulgen-ositive or Feulgen-negative. Comparable results were obtained with methyl reen.

The nuclei in the exo-erythrocytic schizonts were Feulgen-positive but id not stain with methyl green. The nucleus of the merozoite was strongly eulgen-positive and stained with methyl green.

The nuclei in the asexual stages in the erythrocytes were very weakly eulgen-positive. Sometimes the gametocytes exhibited a very faint red olour all over the protoplasm; whether this should be regarded as a true eulgen-positive reaction was difficult to decide. It was, however, clear that he weakest Feulgen-positive reaction in this species was to be found in the tages infecting the erythrocytes.

A positive result for DNA was obtained by the fluorescence method in the ifferent stages of the parasite in the erythrocytes. The merozoites exhibited he strongest reaction for DNA, while in the gametocytes DNA appeared to e present in the least amount.

The microgametes were Feulgen-negative and did not stain with methyl reen.

Plasmodium cynomolgi (fig. 2). The resting nucleus of the early uninucleate öcyst was either faintly Feulgen-positive or Feulgen-negative. The nucleus t this stage was difficult to detect with methyl green. The nuclei in the ajority of the oöcysts remained faintly Feulgen-positive up to the 4th day f infection. In some oöcysts during the same period the nuclei were strongly eulgen-positive.

In the 5th- and 6th-day oöcysts a strong Feulgen-positive reaction was btained in the nuclei. The reacting material in the 4th to 6th-day oöcysts ppeared as minute ring-like structures, which surrounded an unstained zone.

Inclusions of various shapes, very strongly Feulgen-positive, were seen in any advanced oöcysts. Some oöcysts harboured quite a large number of uch inclusions, but lacked the nuclei. Such peculiar oöcysts occurred side by ide with the normal ones. Feulgen-positive inclusions also occurred in the esidual mass of some mature oöcysts.

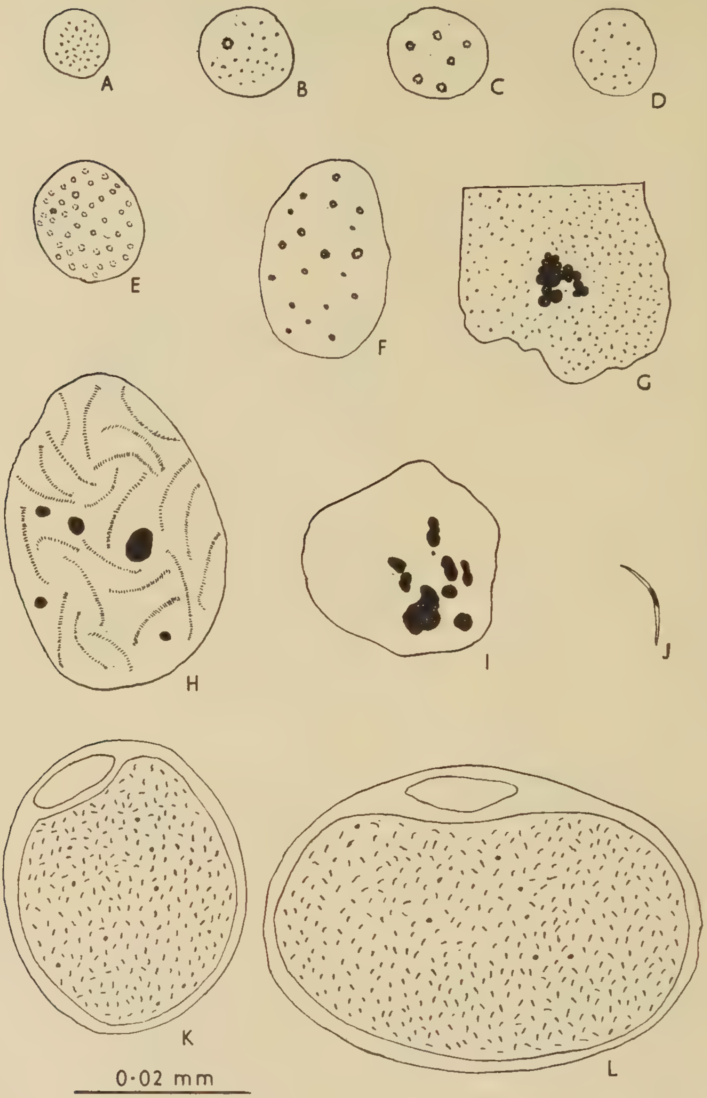
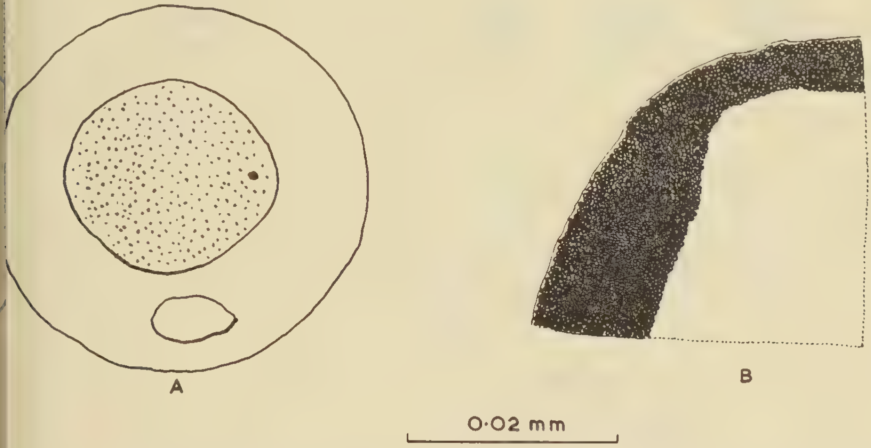


FIG. 2. *Plasmodium cynomolgi.* A, Feulgen-positive nuclei of a 3rd-day oöcyst. B, Feulgen-positive nuclei in a 4th-day oöcyst. One large nucleus with an unstained central core. Rest of the nuclei are small. C, Feulgen-positive reaction in a 4th-day oöcyst. Each nucleus appears to have an unstained central core. D, Feulgen-positive nuclei in a 4th-day oöcyst. E, Feulgen-positive nuclei each with an unstained central core in a 5th-day oöcyst. F, Feulgen-positive nuclei in a 6th-day oöcyst. G, Feulgen-positive inclusions in a 9th-day oöcyst. Normal nuclei appear as small Feulgen-positive dots. H, a 9th-day oöcyst. Feulgen-positive inclusions and Feulgen-positive sporozoite-nuclei. I, Feulgen-positive inclusions in a 9th-day oöcyst. Nuclei are absent. J, Feulgen-positive nucleus of a sporozoite. K, Feulgen-positive nuclei of a pre-erythrocytic schizont. L, the same.

The sporozoites in mature oöcysts had strongly Feulgen-positive nuclei. ch nuclei also stained with methyl green. The nuclei of the sporozoites curring in the salivary gland on the 11th day of infection were usually ongly Feulgen-positive; in some sporozoites during the same period the action was less intense.



3. *Hepatocystis kochi.* A, a schizont in the liver. Feulgen-positive nuclei and a larger ulgen-positive body. B, a part of a merocyst in the liver. Feulgen-positive nuclei at the periphery.

The nuclei in the 6th-, 7th-, and 8th-day pre-erythrocytic schizonts were ulgen-positive. The intensity of reaction often varied in different nuclei the same schizont. These nuclei also stained with methyl green. DNA was tected in the nuclei of these schizonts by the fluorescence method.

The rings, trophozoites, and schizonts in the erythrocytes were usually ulgen-negative, but sometimes a faint Feulgen-positive reaction was noticed them. The reacting material appeared as a small circlet which surrounded unstained region. The nuclei in these stages did not stain with methyl een.

Hepatocystis kochi (fig. 3). The nuclei in the early stage of the parasite seen the hypertrophied cells of the liver were strongly Feulgen-positive and ained with methyl green. In some of these stages a lump of Feulgen-positive aterial was found to occur. This might be an enlarged nucleus or some clusion the significance of which was not clear. The stage of the parasite in liver cells, which showed infoldings of the surface area, had Feulgen-sitive nuclei. The large number of nuclei which occurred between the vy border and the colloid interior of the merocysts were strongly Feulgen-sitive and stained with methyl green. These nuclei also showed yellow orescence (DNA).

The gametocytes were Feulgen-negative and did not stain with methyl een.

Theileria parva (fig. 4). The nuclei in the 'Koch's blue bodies' were strongl Feulgen-positive and stained with methyl green.

Hepatozoon sciuri (fig. 5). The nucleus of the gametocyte was Feulgen-positive and stained with methyl green.

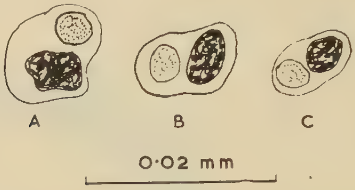


FIG. 4

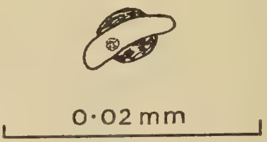


FIG. 5

FIG. 4. *Theileria parva.* A, Feulgen-positive reaction in the nuclei of the Koch's blue body B and C, the same.

FIG. 5. *Hepatozoon sciuri.* A gametocyte in the leucocyte of the host. Feulgen-positiv reaction in the nuclei of the parasite and the host cell.

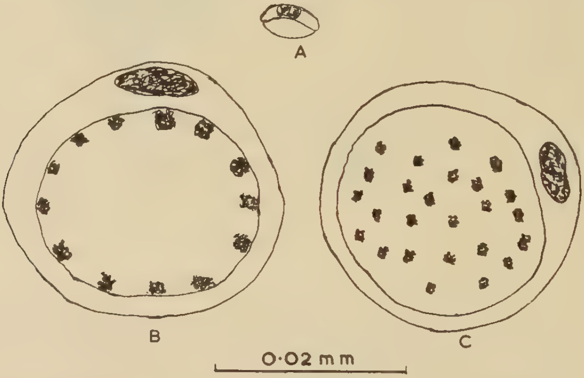


FIG. 6. *Hepatozoon balfouri.* A, Feulgen-positive reaction in the nucleus of the gametocyte B, Feulgen-positive reaction in the nuclei of the schizont in the liver. C, the same.

Hepatozoon balfouri (fig. 6). The nuclei in the schizonts found in the live were Feulgen-positive and stained with methyl green. The nucleus of th gametocyte was Feulgen-positive and stained with methyl green.

Hepatozoon sp. (fig. 7). The nucleus of the gametocyte was Feulgen-positive and stained with methyl green.

Lankesterella sp. (fig. 8). Minute Feulgen-positive granules were found i the nucleus of the sporozoites. These did not stain with methyl green.

Pirhaemocyton sp. (fig. 9). The majority of the stages found in the erythrocytes were Feulgen-negative. In some of these stages the reacting substanc occurred in the form of minute granules.

Eimeria stiedae (fig. 10). The nucleus in the early oöcyst was usuall Feulgen-negative, but in some of these oöcysts a faint Feulgen-positiv reaction was noticed in the nucleus. In one oöcyst a thin rim of the reactin substance was found to surround the nucleus. Minute Feulgen-positiv granules were noticed in the cytoplasm of some of these early oöcysts. Thes

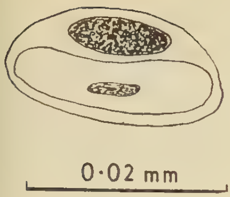


FIG. 7

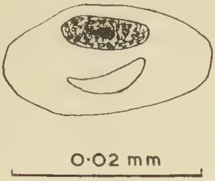


FIG. 8

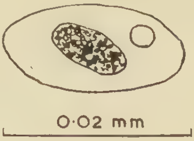
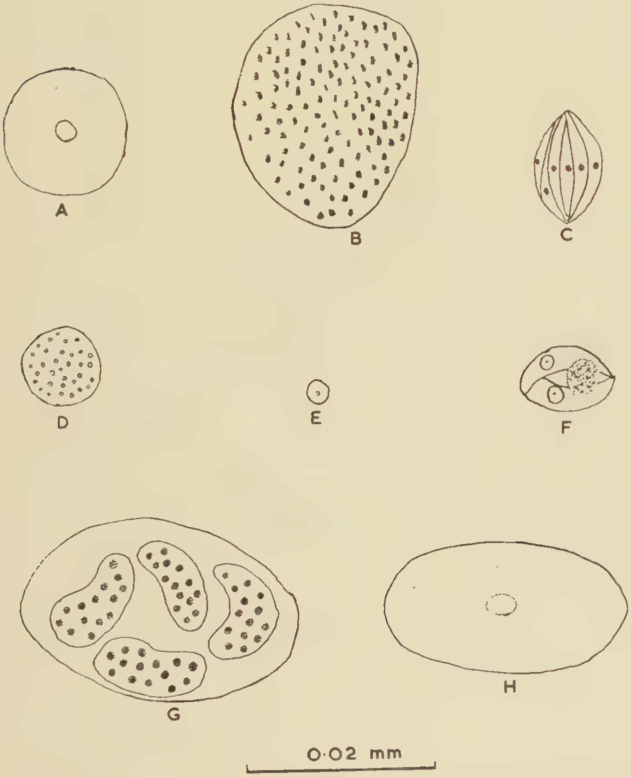


FIG. 9

7. *Hepatozoon* sp. Feulgen-positive reaction in the nucleus of the gametocyte and in that of the host cell.

8. *Lankesterella* sp. Minute Feulgen-positive granules in the nucleus of the sporozoite. Strong reaction in the host nucleus.

9. *Pirhaemocyton* sp. A few minute Feulgen-positive granules in the parasite. The host cell nucleus is strongly Feulgen-positive.



. 10. *Eimeria stiedae*. A, a female gametocyte. The periphery of the nucleus is Feulgen-
tive. A few Feulgen-positive granules seen in the cytoplasm. B, a male gametocyte.
lgen-positive reaction. C, Feulgen-positive nuclei of the merozoites. D, Feulgen-positive
ei in a schizont. E, Feulgen-positive nucleus of a trophozoite. F, Feulgen-positive granules
ne nucleus of the sporozoite. Feulgen-positive material in the residual body. G, sporo-
ts containing Feulgen-positive bodies. H, early oöcyst. Peripheral layer of the nucleus
lgen-positive. This layer contains one deeply-stained dot at one side. Minute Feulgen-
positive particles occur in the cytoplasm.

granules could not be seen by methyl green. DNA was detected by th fluorescence method in a number of unidentified bodies in these oöcysts.

In each sporoblast a number of Feulgen-positive structures occurred These probably represented the nuclei. A minute Feulgen-positive granul occurred at the centre of the nucleus of the two sporozoites in the sporocys and a large number of Feulgen-positive granules occurred in the residua mass.

The nucleus was faintly Feulgen-positive in the trophozoites found in th cells of the bile ducts. The reacting material appeared in the form of a minut ring. With the methyl green stain the nucleus of the trophozoite could no be detected. In the nuclei of the schizonts the pattern of Feulgen reaction wa similar. The nucleus of the merozoite was Feulgen-positive and also staine with methyl green. The male gametocyte had Feulgen-positive nuclei. Th male gametes were Feulgen-positive. The presence of DNA in these stage was also demonstrated by the fluorescence method. The nucleus of th female gametocyte was Feulgen-negative. Close observation revealed th presence of minute Feulgen-positive granules in the cytoplasm of the femal gametocytes.

DISCUSSION

In the present investigation Feulgen-positive inclusions have been foun to occur in certain stages of *Plasmodium*, *Hepatocystis*, and *Eimeria*. Thoug DNA is known to be chiefly a constituent of the nucleus, in rare instances i has been reported to occur in the cytoplasm of some Protozoa. It has bee shown by various workers that the distribution of such extranuclear DN may coincide with specific cytoplasmic organellae. Such cytoplasmic organellae include the kinetoplasts of various species of *Trypanosoma* (Bresslau an Scremin, 1924; Jírovec, 1927; Robertson, 1927; Roskin and Schischliaiewa 1928; and Lillie, 1947), *Herpetomonas culicidarum* (da Cunha and Muniz 1928), *Leishmania tropica* (Roskin and Romanowa, 1928), *L. donovani* (Se Gupta and others, 1953), the 'chromidial net' in *Patellina* (Meyers, 1935) the chromatoid bodies in the cysts of *Entamoeba histolytica* (Ray and Se Gupta, 1954), the basal granules of the cilia of *Balantidium coli* (Sen Gupt and Ray, 1955), and certain trichocysts of *Polykrikos schwartzi* (Hovasse 1951). Feulgen-positive material has also been reported in the cytoplasm o 10% of the oöcysts of *Eimeria tenella* (Ray and Gill, 1955).

The most conspicuous of the Feulgen-positive inclusions, reported in th present communication, are those found in the oöcysts of *Plasmodium cynomolgi* and *P. gallinaceum*. It appears probable that these occurrences represen degenerating changes. Such degeneration might be total or partial. In the cas of total degeneration the entire oöcyst is full of lumps of Feulgen-positiv material and there is no trace of nuclei. In the case of partial degeneration th inclusions occur side by side with the normal nuclei. In some mature oöcyst the inclusions can be seen along with the sporozoite nuclei. It is not clea whether these inclusions should be regarded as a normal occurrence in th cysts or as an unusual phenomenon. The possibility can be ruled out that ch inclusions might develop in all the oöcysts at a certain stage, because e oöcysts with inclusions and those without inclusions are found at the same ge of maturity. It does not appear likely that the oöcysts without inclusions e the abnormal ones, since the majority of the oöcysts that mature are with- t such inclusions. It should be emphasized here that there is no evidence to ate the formation of Ross's black spores with the type of degeneration sociated with the Feulgen-positive inclusions reported in this communica- n.

The presence of minute Feulgen-positive particles in the cytoplasm of the crogametocytes and some early oöcysts of *Eimeria stiedae* is another eresting phenomenon. It is difficult to believe that these Feulgen-positive rticles represent degenerating changes, as these occur widely in a large mber of the parasites, in which no cytological change could be found.

It should be recalled in this connexion that the occurrence of DNA in the toplasm has been reported in certain lower organisms, and such DNA terial has been compared with infectious agents. Lwoff (1952) described rticles containing DNA, the 'pro-virus particles', in certain lysogenic cteria. The 'killer' races of *Paramecium aurelia* possess the 'Kappa' factor the cytoplasm, which contains DNA (Preer, 1950). It might be argued that e Feulgen-positive material occurring in the cytoplasm of the stages of the rasite studied here represents elements of viral or bacterial nature which ve found their way into the parasites; but the evidence available at the ment is insufficient to sustain this view. A survey of the literature, however, eals that a case of bacterial inclusion in the oöcysts of *Eimeria labbeana* has en recorded (Yakimoff and Timofeeff, 1940).

The presence of a large number of Feulgen-positive bodies in the sporo- sts of *E. stiedae* is difficult to explain. Since each sporoblast is destined to e rise to two sporozoites only, the occurrence of a large number of nuclei uld be anomalous. It does not appear likely that these Feulgen-positive dies represent anything else than nuclei. We do not know of any cytoplasmic ucture which would react in this way during the progress of sporogony. analogy with other Sporozoa studied here, it might be expected that clei would not be Feulgen-negative at this stage. If these bodies do not present the nuclei, then the failure of the Feulgen reaction to detect the clei at this stage will pose a new problem. It may be argued that these dies represent true nuclei, the total number of which may correspond to e number of the sporozoites in the oöcyst of a hypothetical ancestral form. may be argued that in the course of evolution there has been a reduction the number of sporozoites, that the number of nuclei in the sporoblast in cess of the total number of sporozoites to be formed in an oöcyst is destined go to waste, and that the remainder are perhaps represented by the Feulgen- sitive granules in the residual body. It is, however, clear that the evidence ilable at the moment is inadequate to sustain such a theory. Future in- stigation will perhaps throw light on the subject. It is interesting to notice that this particular feature of the sporoblasts was not present in Patillo an Becker's (1955) material.

In the course of the present investigation a Feulgen-negative reaction ha been noticed in some stages of Sporozoa. It is difficult to explain the total lac of detectable DNA in the gametocytes and the male gametes of *Plasmodium* and in the early gametocytes of *Eimeria*. It is to be expected that these stage would carry the hereditary determinants, of which DNA is a major constituent. It may be possible that the quantity of DNA present in these stage of the two parasites is small and in highly dispersed condition, and henc undetectable. It is also to be noted that some of these stages of the two parasites appeared to show the presence of DNA by the fluorescence metho Under these circumstances it would appear that a Feulgen-negative reactio did not necessarily mean the absence of DNA.

In the course of the present investigation the Feulgen reaction in th sporozoites did not yield uniform results. Some were strongly Feulgen-positive, while some were Feulgen-negative. It is difficult to explain thi difference. It remains to be established if ageing or some other factor is to b taken into consideration while interpreting the results of the Feulgen reaction. A survey of the literature reveals that there are some previous report about the influence of certain factors on the result of the Feulgen reaction i Protozoa. For example, a diminution of DNA in the macronucleus of *Paramecium aurelia* was found to ensue as a result of starvation, lack of oxyge and exposure to low temperature (Gromova, 1941). The clumping of th nuclear material and the loss of Feulgen-positive appearance in the crithidia of *Trypanosoma melophagium* was ascribed to death and degeneration (va Thiel, 1925).

The question whether the Feulgen reaction in Protozoa can be used as guide in differentiating one species from another, appears to be relevant i the light of the present study. Singh (1952) sought to classify the orde Amoebida on the basis of the Feulgen reaction in the nuclei of 9 species o free-living amoebae. Pan and Geiman (1955) thought that the Feulgen reaction might be used to differentiate *Entamoeba histolytica* from *E. coli*. Bra (1957*c*) emphasized a dissimilarity in the outcome of the Feulgen reactio in the pre-erythocytic schizonts of *Plasmodium cynomolgi*, *P. ovale*, an *P. inui*. In the former species the Feulgen reaction was said to be negativ except in the later stages, while in *P. ovale* and *P. inui* DNA could be easil detected by the Feulgen reaction.

In the course of the present study a close resemblance in the outcome o the Feulgen reaction was noticed in the corresponding stages of *P. gallinaceum* and *P. cynomolgi*. In *Hepatocystis kochi* the result was similar. The tissu stages of *Theileria parva* were Feulgen-positive and in that respect broadl resembled the corresponding stages of the malarial parasite. In the thre species of *Hepatozoon* studied here the gametocytes were Feulgen-positive the intensity of reaction being the same so far as the visual estimation of colou is concerned. The sporogony stages of *H. sciuri* showed a transition from th lgen-negative stage to a Feulgen-positive stage (Dasgupta and Meedeniya, 8), and in that respect resembled the corresponding stages of *Plasmodium*. t is important to recognize here that the Feulgen-positive gametocytes of *patozoon* stand in sharp contrast to the Feulgen-negative gametocytes of *smodium* and *Hepatocystis*. This is an example of a group of Sporozoa ch differs from another group of Sporozoa in the cytochemically-recog-able intracellular content of DNA at a specific stage of life.

n *Eimeria stiedae* it was found that the nuclei in the asexual cycle were lgen-positive, while those of the sexual cycle exhibited sexual dimor-sm; that is to say, the nuclei of the microgametocyte and the microgamete e Feulgen-positive, while those of the macrogametocyte and the macro-nete were Feulgen-negative. The fact thus seems to be established that re exists a sharp difference of cytochemically-recognizable DNA in the netocytes of three major groups of Sporozoa.

Plasmodium and Hepatocystis	*Hepatozoon*	*Eimeria*
gametocytes Feulgen-egative	All gametocytes Feulgen-positive	♂ Feulgen-positive ♀ Feulgen-negative

s it possible, then, to determine the relationship between different species the basis of the Feulgen reaction in the gametocytes? A conclusive answer his question appears to be difficult. It is realized that certain broad facts revealed by such a study, but any attempt to interpret these results in erentiating one group of Sporozoa from another should be made with reme caution. The contradiction between the results of the present in-tigation and that of Bray (1957*c*) on the Feulgen reaction in the pre-throcytic schizonts of *P. cynomolgi* only serves to emphasize the fact that ndividual instances the parasites of the same species are liable to vary in way or another, which may impair the diagnostic value of the Feulgen tion at a specific age or in a specific stage of the life-cycle.

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A Histochemical Investigation of the Pituitary Glands of some Teleost Fish

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With two plates (figs. 2 and 3)

SUMMARY

Some histochemical observations on the pituitaries of three marine and three fresh-ater teleost fish have been made. It has been shown that cells of this gland, which re stained identically in different species by Azan, do not always give identical histohemical reactions, and that in the same species cells which after histological staining ave similar tinctorial affinities may be differentiated histochemically. These observaons are discussed with regard to the function of the gland.

INTRODUCTION

THE histology of the pituitary of teleost fish has been described by a number of authors (Kerr, 1942 *a*, *b*; Green, 1951; Olivereau, 1954 *a*, *b*), ut these descriptions have added little to the knowledge of the histophysioogy of the gland and little is known of the sites of hormone production. istological studies have indicated that the so-called basiphils or cyanophils re concerned with the elaboration of the gonadotrophic hormone (Rasquin, 949; Scruggs, 1951), while a second cyanophil type has been identified as thyrotroph in *Astyanax mexicanus* (Atz, 1953) and in *Phoxinus laevis* Barrington and Matty, 1955). Both basiphils and acidophils have been mplicated in the release of ACTH by the teleost pituitary (Rasquin and Stoll, 955; Chavin, 1956), and acidophils with the release of somatotrophin Olivereau, 1954*b*). However, except for the use of the periodic acid/Schiff PAS) reaction there has been no histochemical investigation of the fish denohypophysis. Furthermore, as it has recently been shown that the cidophils of the pituitary—generally assumed to be PAS-negative—may be PAS-positive in fish (Matty, 1957), it was thought that a general cytochemical nvestigation of the pituitaries of a number of teleost fish was desirable in rder to provide an additional basis for further investigations of the histohysiology of this gland.

MATERIALS AND METHODS

The pituitaries of three marine teleost fish, the rainbow parrot fish *Pseudocarus guacamaia*, the mud-belly *Scarus croicensis*, and the Bermuda white runt *Bathystoma aurolineatum*, and three fresh-water fish, the perch *Perca luviatilis*, the roach *Rutilus rutilus*, and the minnow *Phoxinus laevis* were

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examined. Glands from all the above fish were placed in formaldehyde-saline and in Heidenhain's mercuric chloride / formalin. Other fixatives employed will be referred to later. Material from the minnow was decalcified in 5% versene (Schajowicz and Cabrini, 1956). After overnight fixation and washing in running water, next day both paraffin and gelatin sections were prepared and examined after histochemical and routine histological staining methods (Heidenhain's Azan and Heidenhain's iron haematoxylin with orange G counterstain). In addition the aldehyde-fuchsin method of Gomori (Halmi, 1952), the chrome alum / haematoxylin and phloxine method of Gomori as modified by Bargmann and Hild (1949), and the pyronin / methyl green method for RNA (Brachet, 1953) were applied.

The following histochemical methods were used:

Method for the detection of carbohydrates

PAS test (McManus, 1946) with the acetylation technique of McManus and Carson (1950). In addition to the fixatives quoted above, material fixed in Bouin was also examined by this method.

Methods for the detection of amino-acids

Demonstration of tyrosine by a modification of the Morel–Sisley diazotization procedure (Lillie, 1957).

Millon's test for tyrosine as described by Pearse (1953).

Demonstration of cystine and cysteine by the performic acid / Alcian blue method (Adams and Sloper, 1956).

Thioglycollate-ferricyanide reaction for sulphydryl groups (Adams, 1956).

Dihydroxydinapthyldisulphide (DDD) reaction for disulphide and sulphydryl groups (Barrnett and Seligman, 1954).

Coupled tetrazonium reaction of Danielli (1947, 1950), the technical details being those described by Pearse (1953).

Iodination / coupled tetrazonium reaction for histidine (Landing and Hall, 1956).

Sakaguchi's 1925 test for arginine, according to the method adapted to histochemical use by Baker (1947).

Methods for the detection of lipids

Gelatin sections fixed as above and also after formaldehyde-calcium and postchroming (Baker, 1949). Some sections were stained in Sudan black B and others in Fettrot (Pearse, 1953).

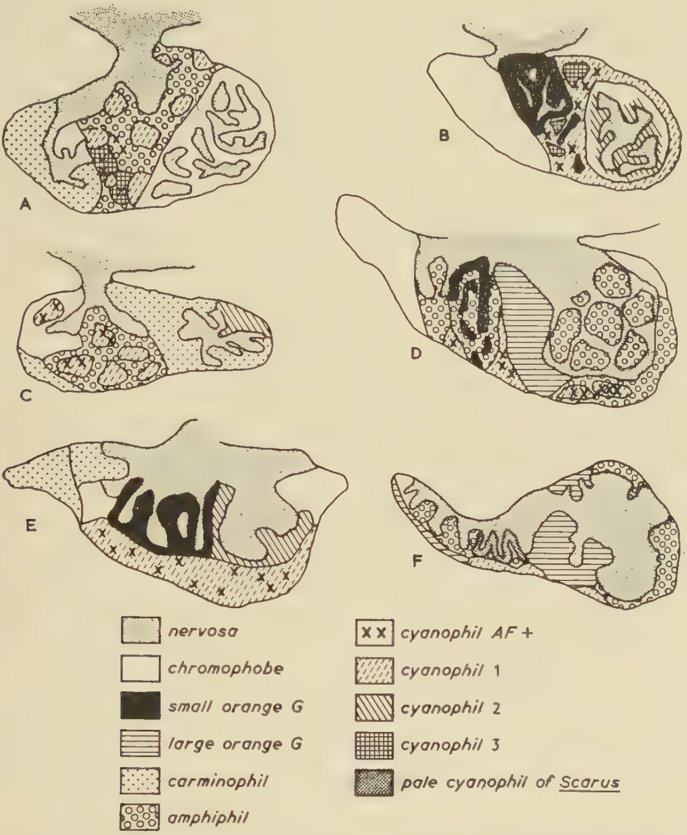
Baker's (1946) acid haematein and pyridine extraction test for phospholipids.

Copper phthalocyanin method for phospholipid according to Klüver and Barrera (1953).

OBSERVATIONS

As the microanatomy of the pituitaries of *Pseudoscarus guacamaia*, *Scarus croicensis*, and *Bathystoma aurolineatum* has not been described, their structure after Azan staining must be first outlined (fig. 1). None has a definite stalk;

it is, they are of the platybasal form, and in shape all are similar to that of e perch (fig. 1, E), having an elongated anterior region and a rounded sterior region. The neurohypophyses are well developed, and in all three ecies the adenohypophysis, which may be divided conventionally into the



G. 1. Diagrams of median longitudinal sections through the pituitary glands of: A, *Rutilus tilus*; B, *Bathystoma aurolineatum*; C, *Phoxinus laevis*; D, *Pseudoscarus guacamaia*; E, *Perca viatilis*; F, *Scarus croicensis*; showing the different cell types after staining with Azan. The stribution of aldehyde-fuchsin positive (AF+) cyanophils (thyrotrophs) is also superimposed on these diagrams.

sterior, median, and anterior glandular zones, surrounds and interdigitates ith the neurohypophysis.

Both *Scarus* and *Pseudoscarus* have an anterior glandular zone comprised a cap of small chromophobes which have immediately posterior to them region of small amphiphils which contain little cytoplasm. In addition *carus* has an anterior-ventral layer of small cells that stain with orange G. he median glandular zone in *Pseudoscarus* consists dorsally of cells staining ightly with orange G; these form a continuous sheet at the boundary with the neurohypophysis and intermingle ventrally with amphiphils. Along the latero-ventral border of this zone are found patches of cyanophils (the basiphils ('basophils') of earlier authors), of two kinds; small cells staining lightly with aniline blue and larger deeply staining cells with slightly granular cytoplasm and large vacuoles. The posterior half of this zone is composed of very large polygonal orange G cells interspersed with a few very small amphiphils. The median glandular region of *Scarus* is similar except that the sheet of cells at the anterior dorsal border are weakly cyanophil; they do not stain brightly with orange G. Both species have similar posterior glandular zones consisting of small amphiphils and a few dull blue cyanophils (fig. 1, D, F).

Bathystoma has a well-marked large anterior glandular zone composed of chromophobes. The median glandular zone has sheets of deeply staining orange G cells adjacent to the neurohypophysis; these extend as strands into the more ventral, predominantly cyanophil region. The cyanophils are of two well-defined types. Patches of cells staining a very light blue lie amongst cells with a deep blue granular cytoplasm; the latter extend posteriorly round the outer edge of the adenohypophysis. The posterior glandular zone is partially enclosed by these deep blue cyanophils of the median glandular zone, which itself encloses the posterior ramifications of the neurohypophysis. The cells lying against the neurohypophysis are weakly staining cyanophils, and between these cyanophils and the peripheral median glandular cyanophils is found a region of chromophobes (fig. 1, B).

The histology of the pituitaries of *Phoxinus*, *Rutilus*, and *Perca* has been described in a similar manner by Kerr (1942). Diagrams of the glands of all 6 species after staining with Azan are shown in fig. 1, and hereafter the nomenclature of the figure will be used in the text. The type 3 cyanophils of *Rutilus* and *Bathystoma* are identified as islands of small pale blue AF-negative cells, but this does not preclude their possible occurrence in other animals examined where small cyanophils appear scattered among larger areas of type 1 cyanophils, nor is it impossible that these type 3 cells are precursors of the type 1 cells.

Aldehyde-fuchsin reaction. The distribution of cells positive to aldehyde-fuchsin (AF-positive cells) has already been described for the minnow pituitary, and these cells have been identified as TSH-secreting (Barrington and Matty, 1955). Similar cells are identified in all other teleost glands so far examined and their distribution is shown in fig. 1. These cells are always cyanophil and PAS-positive, and are characteristic of the median glandular zone. Furthermore in *Pseudoscarus* these cells seem to be invariably vacuolated (fig. 2, A). In *Rutilus* it appeared to be this type of cell in which the cytoplasm became vacuolated and nucleus pycnotic after immersion in the anti-thyroid drug, thiourea (fig. 2, B). This observation was made on two groups of fish that had been immersed in a 0·1% thiourea solution for periods of 53 and 151 days and provides further evidence that it is the AF-positive cell of the median glandular zone of the teleost pituitary which is responsible for the production of TSH.

Chrome alum / haematoxylin and phloxine procedure. The neurosecretory terial of the neurohypophysis was stained by this procedure in all species amined. In all pituitaries, however, there appeared to be a marked tendency this material to be concentrated in that part of the neurohypophysis ich is in intimate contact with the posterior glandular zone of the adenopophysis; little chrome-haematoxylin positive substance was ever found the nervosa lying between the cells of the anterior glandular zone.

The type 1 cyanophils of all the species were chrome-haematoxylin positive g. 3, D, opposite p. 263), and in *Rutilus* the occasional cyanophil found in anterior glandular zone of carminophils also stained by this procedure. wever, the type 2 cyanophils of the posterior glandular zone of *Phoxinus*, *rca*, and *Bathystoma* were not stained by this procedure. The AF-positive nophils of all species were intensely chrome-haematoxylin positive.

Pyronin G / methyl green procedure. RNA was present in the nucleoli of all ls of the adenohypophysis but few of the different Azan cell types showed y visible difference in amounts of extra-nucleolar RNA or in nucleolar size. *Rutilus*, however, the chromophobes of the posterior glandular zone owed RNA concentrated in one or more eccentric patches outside the clei in greater amounts than is found elsewhere in this pituitary (fig. 2, C). so the small orange G cells of *Perca* showed evidence of a higher RNA ncentration than other cell types.

Carbohydrates. Both the AF-positive cyanophils and the type 1 cyanophils the median glandular zone of *Rutilus*, *Phoxinus*, and *Perca* were strongly S-positive. The AF-positive cells showed a fine magenta granulation sely embracing the nucleus after the PAS procedure, whereas the type 1 ls contained coarse granules of PAS-positive material scattered throughout cytoplasm. In *Rutilus* there were also a few lightly staining cyanophils of anterior glandular zone which were PAS-positive, whereas in *Phoxinus* d *Perca* a third group of PAS-positive cells were the type 2 cyanophils of posterior glandular zone. In *Bathystoma* the cyanophils which morphogically and topographically resemble the type 1, type 2, and AF-positive ls of the above species were also PAS-positive, but in addition the cyanoils of the median glandular zone which appear in patches between the pe 1 cells and the AF-positive cells also reacted to the PAS procedure. In ese four species all the cyanophils were PAS-positive and no other cell of adenohypophysis appeared to contain carbohydrate.

In *Pseudoscarus* the AF-positive cyanophils and the AF-negative cyanoils which surround them were PAS-positive, the former staining deeply. so the large orange G cells of the median glandular zone were PAS-positive. nally, in *Scarus* the large orange G cells were PAS-positive, but the cyanoils of the median glandular zone adjacent to the nervosa were PAS-negative. e amphiphils of the posterior glandular zone were also PAS-positive, but mewhat weakly so; this cell type may correspond to the type 2 cell of other ecies.

The nervosa in all fish examined was PAS-negative.

Tyrosine. After applying the Morel–Sisley diazotization procedure it was found that a strong reaction can be obtained in certain cell types. The small orange G type cells of *Perca* showed intense orange-red coloration whereas no other type in this species showed other than the general faint pink-orange background reaction. The small orange G cells of *Bathystoma* and *Pseudoscarus* and the weakly cyanophil cells of the median glandular zone of *Scarus* also gave an orange-pink coloration after this technique, but it was not as intense as that seen in *Perca. Rutilus* and *Phoxinus* also have a cell type which gave a much stronger reaction than any other type but in these pituitaries it is shown by making careful comparison with adjacent sections stained in Azan that it was the cyanophil type 1 cell (fig. 2, E). Thus all the pituitaries examined appeared to have one cell type which has a high tyrosine content, although after Azan staining these cells may differ tinctorially.

The Millon reaction did not colour the cells as intensely as the above method and its localization and the differentiation of cell types after this technique was difficult to interpret.

Performic acid / Alcian blue reaction. Cystine and/or cysteine was localized by a deep blue coloration in the cytoplasm of the cyanophil type 1 cells of all the fish that were examined. In *Rutilus* and *Phoxinus* there was a pale blue granular colouring of the other cyanophil types. In *Perca* the cyanophil type 2 cell, which can clearly be differentiated histologically, gave a definite blue coloration and a somewhat weaker reaction was seen in the small orange G cells (fig. 3, B). The neurosecretory material of the neurohypophysis in all species gave a positive but variable reaction.

Thioglycollate-ferricyanide reaction. Sulphydryl groups were demonstrated by a deep blue colour after this reaction. The cyanophil type 1 of *Rutilus*, including the scattered cyanophils of the anterior carminophil zone, reacted intensely but a weaker reaction was shown by other cyanophils (fig. 3, A). In *Phoxinus* both cyanophil 1 and 2 gave positive reactions. This was also true of *Perca* but in this case the reaction in the cyanophil type 1 was not strongly marked and the small orange G were slightly positive. In *Pseudoscarus* and *Scarus* the large orange G cells gave a pale but distinct reaction. Again the neurosecretory material gave a positive but variable reaction.

Dihydroxydinapthyldisulphide reaction. The results obtained by this method were in agreement with the thioglycollate-ferricyanide reaction but with some minor quantitative differences. For example, a strong reaction colouring both the small orange G cells and the 'sphere cell' droplets of *Perca* a deep brown was given after applying this method, but both cyanophil type 1 and 2, although positive, were coloured only a light brown (fig. 3, C). The cyanophil

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FIG. 2 (plate). Photomicrographs of pituitaries of: A, *Pseudoscarus*, showing normal vacuolated AF+ cells; B, *Rutilus*, with a large vacuolated 'thyroidectomy' cell with a pycnotic nucleus (*n*); C, *Rutilus*, showing presence of RNA in posterior glandular region cells; D, *Pseudoscarus*, with PAS+orange G cells (*og*+), PAS−orange G cells (*og*−), and PAS+throtroph (*t*); E, *Phoxinus*, with tyrosine-positive cyanophil 1 (*c*1) and tyrosine−negative orange G cell (*og*); F, *Perca*, with histidine positive small orange G cells (*og*).

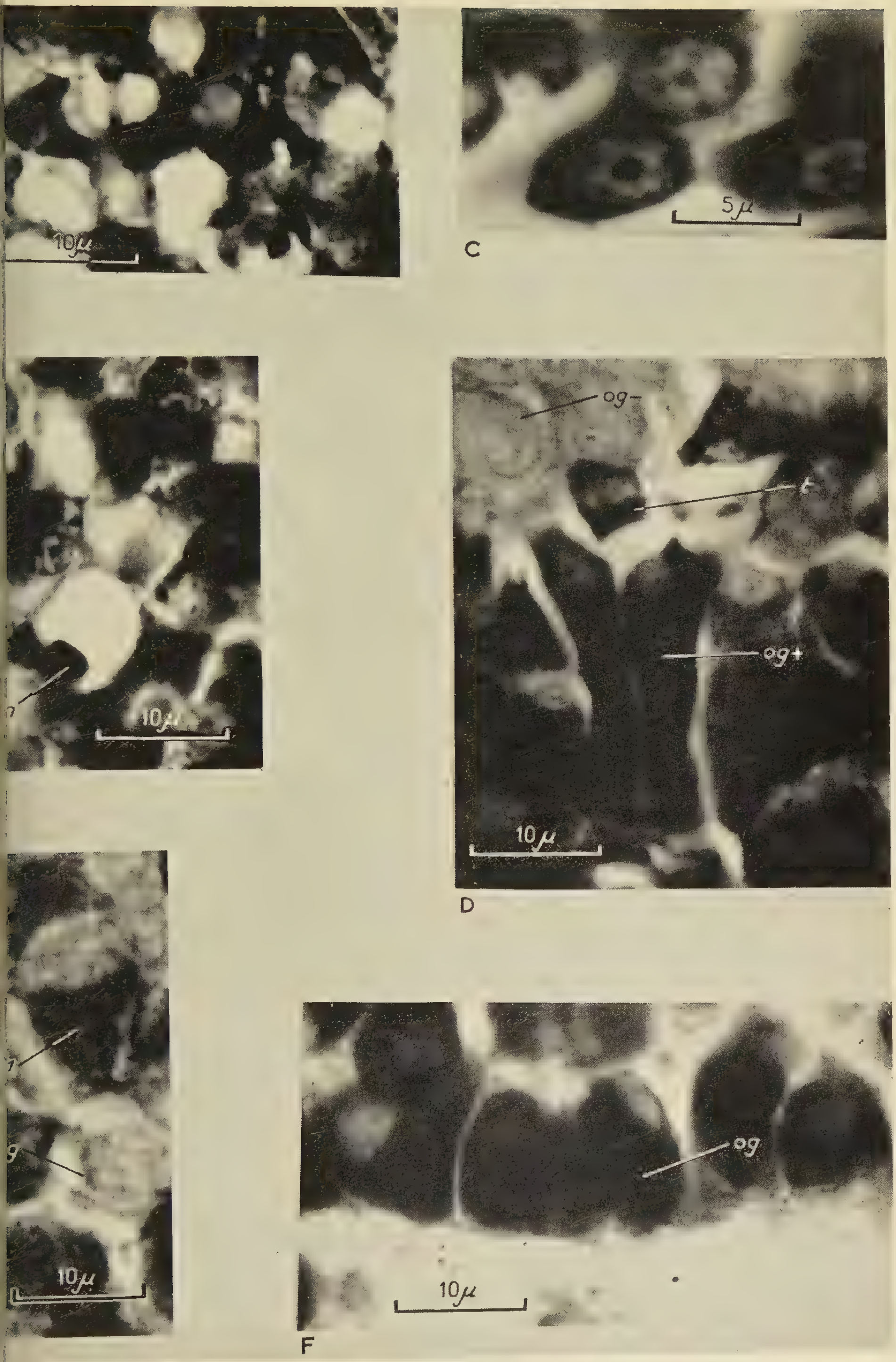

FIG. 2

A. J. MATTY *and* J. M. MATTY

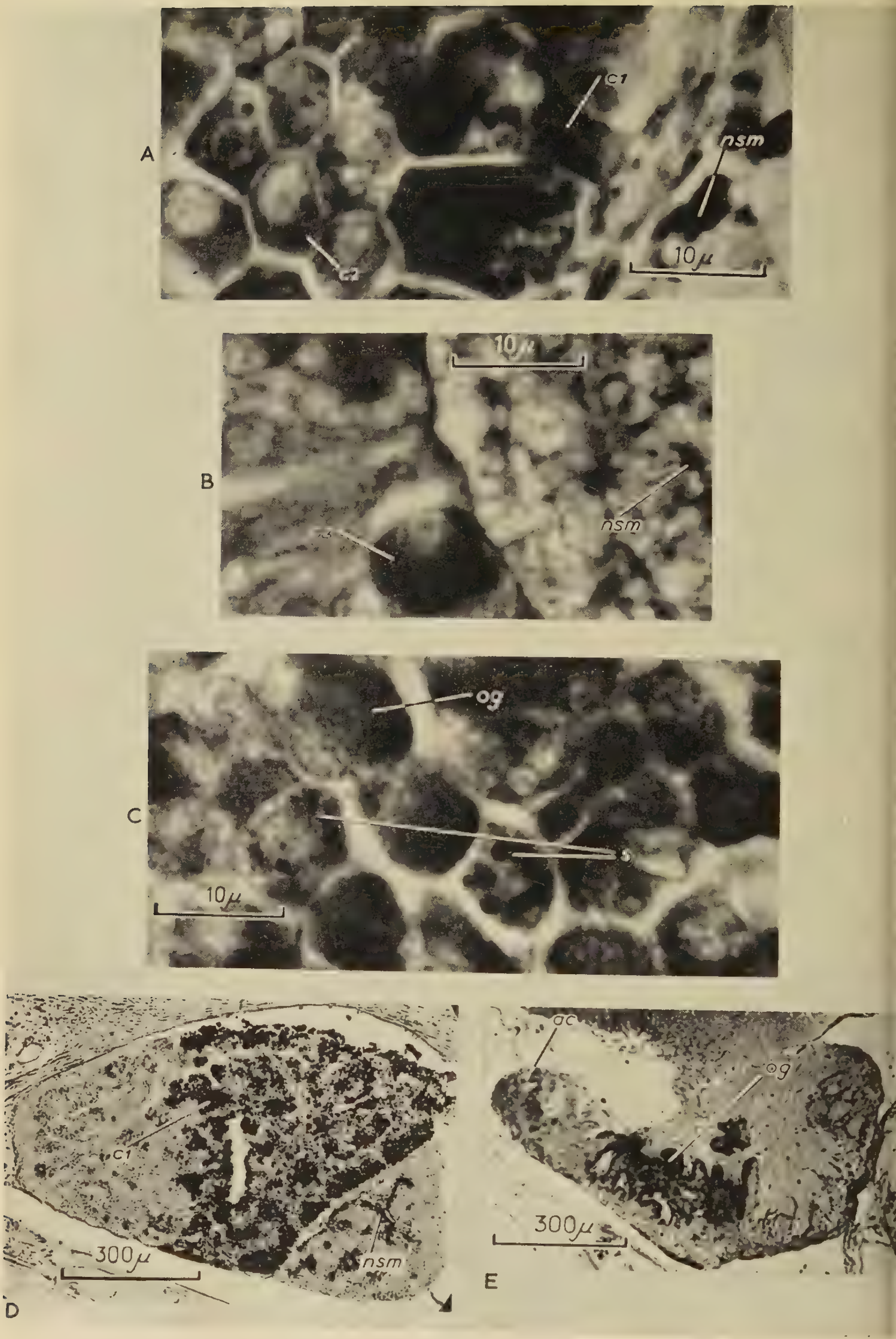

FIG. 3

A. J. MATTY *and* J. M. MATTY

›e 1 cells again were positive in all species and again the other cyanophils *Rutilus* demonstrated the presence of —SH or S—S groups. The cyanophil ›e 2 in *Phoxinus*, *Perca*, and *Bathystoma* gave pale but positive reactions. ıe cytoplasm of the large orange G cells of *Pseudoscarus* and *Scarus* contain ›re DDD-positive material than any other cell type in these pituitaries. The all orange G cells of *Pseudoscarus* are weakly positive. The neurosecretory terial was unreactive.

Coupled tetrazonium reaction. The brown coloration indicating the presence histidine, tryptophane, or tyrosine was variable in intensity. In *Rutilus* and *thystoma* the cyanophil type 1 gave a definite positive reaction, although t all the cyanophil type 1 of *Phoxinus* were coloured. The cyanophil type 2 ls of both *Phoxinus* and *Bathystoma* were stained deep brown. The small ınge G cells gave a strongly positive reaction in *Perca*, *Bathystoma*, and *udoscarus*, although somewhat paler in the latter where the large orange G ls showed the strongest reaction. The large orange G cells of *Scarus* were o positive. In all species examined the nervosa gave a positive but variable ponse.

Iodination / coupled tetrazonium reaction for histidine. This method gave an er-all background coloration to all the pituitaries stained, and only in *Perca* d *Bathystoma* was there any positive reaction. In both species the small ınge G cells were strongly positive and in *Perca* the anterior cap of carmiphils also gave a definite positive reaction, while the cyanophil type 2 cells re slightly stained (fig. 3, E). In all species the nervosa remained unstained.

Sakaguchi's (1925) test for arginine. In no cell in any pituitary examined was possible to demonstrate any concentration of arginine.

With reference to the observations on amino-acids quoted above it was not ssible to differentiate AF-positive cyanophils always from surrounding ›e 1 cyanophils after these histo-chemical procedures, and we therefore ve been unable to make specific references.

Lipids. Staining gelatin sections with Sudan black B or Fettrot gave no sitive indication of the presence of fat-droplets in any pituitary cells or in nervosa. However, after Sudan black the nervosa appeared blue, which ıy have indicated a very fine deposit of fatty material. Neither the neuroretory substance or any adenohypophysial cells showed a marked concention of phospholipid after acid haematein. The copper phthalocyanin ction, however, although not demonstrating any structures corresponding Gomori-positive material, did stain with varying intensity both nuclei and toplasm of adenohypophysial cells in all species. In view of the negative

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IG. 3 (plate). Photomicrographs of pituitaries of: A, *Rutilus*, with thioglycollate-ferricyanide itive cyanophils type 1 and 2 (*c* 1 and *c* 2) and neurosecretory material (*nsm*); B, *Perca*, with formic acid / Alcian blue positive cyanophil 2 (*c* 2) and neurosecretory material (*nsm*); *Perca*, with DDD—positive small orange G cells (*og*) and 'sphere cells' (*s*); D, *Rutilus*, gitudinal section showing chrome-haematoxylin positive cyanophil (*c* 1) and neurosecretory erial (*nsm*); E, *Perca*, longitudinal section showing presence of histidine in anterior minophils (*ac*) and in small orange G cells (*og*) of the median glandular zone.

Sudan black and acid haematein results, the copper phthalocyanin reaction must be suspect.

DISCUSSION

The problems of homologizing the zones of the teleost pituitary with those of other vertebrates and the dependent problem of correct terminology for these zones has exercised investigators for many years (Stendall, 1914; Pickford and Atz, 1957). Kerr (1948) has suggested that the anterior glandular region and the median glandular region of the adenohypophysis (the pro- and meso-adenohypophysis of Pickford) are homologous with the pars distalis of higher vertebrates and the posterior zone with the whole or part of the pars intermedia. Reviews by Charipper (1937) and Pickford and Atz (1957) have demonstrated how extensively the histology of the teleostean pituitary has been studied and how variable have been the descriptions of cell types and their distribution in the gland. The gland is probably highly labile in form, as previous authors have suggested, but much of the apparent variance is possibly due to the describing of pituitary cells after the use of different stains. In the present work the glands were examined histologically after staining with the Azan method, for the reason that this triple stain gives clear, non-fading, repeatable results and differentiates a number of cell types in the teleost pituitary, and furthermore has been used in a number of the most recent morphological accounts of this gland. However, it is fully realized that other staining methods might have given a somewhat different tinctorial picture which could equally well have been used to give a histological description for comparison with the histochemical investigation.

Although most of the cell types found in the mammalian pituitary have been identified in teleosts, only two types have been related directly to specific hormone production, namely the thyrotrophs and gonadotrophs, which are cyanophils of the median glandular region (Pickford and Atz, 1957). Even here much of the evidence is circumstantial and the FSH cell has not been distinguished from the LH cell, although Olivereau (1954*b*) has associated the acidophils of the pituitary of *Salmo salar* with the luteinizing hormone, a view for which there is little evidence in mammals and even less in fish. As in mammals the somatotroph has been assumed to be an acidophil but the evidence, based upon observations of pituitaries during development and growth, is casual, and recently Olivereau and Francotte-Henry (1956) noted that the acidophils of *Caecobarbus geertsi* may be abundant when growth is slow. Other cells, both of the anterior and median glandular zone, undergo changes during growth, seasonally, and during migratory periods, but these observations (reviewed by Pickford, 1957) have not demonstrated specific hormone-producing sites.

The evidence for a site of ACTH production, i.e. a corticotroph, is confused. Rasquin and Stoll (1955) have claimed that a basiphil which was not a gonadotroph (although no evidence for this statement was given) produced ACTH; while Rasquin and Atz (1952) and Chavin (1956), after treating fish th saline, DOCA, or ACTH, have demonstrated changes in both the acido- ils and basiphils of the median glandular zone. Intermedin, or the melano- ore-dispersing hormone, is certainly produced in the posterior glandular ne of the teleost adenohypophysis (Hewer, 1926), but again the cellular site this hormone has not been revealed and Chavin (1956) could find no change any cells of this zone after treatment with ACTH and intermedin.

Argyrophils have been associated in the mammal pituitary with ACTH- lease or gonadotrophin production (Knigge, 1955 *a*, *b*), but have hitherto ot been described in teleost fish. However, during this study it was found at cyanophils of the posterior glandular region of the adenohypophysis of *hoxinus* were argyrophil after applying the method of Ranson as modified y Smith (1956) or Bodian (1936), whereas the weakly staining amphiphils chromophobes were not. Also a clearly marked arygyrophil is found in *seudoscarus*, but here it is identified as the large deeply staining vacuolated yanophil of the posterior-ventral region of the median glandular zone.

Although it has been shown in the present work that the teleost neuro- ypophysis contains neurosecretory material which is histochemically similar that of the rat (Howe and Pearse, 1956), functionally the neurohypophysis an enigma. No 'water balance principle' has been demonstrated (Fontaine, 956), nor have studies using mammalian posterior pituitary hormones re- ealed that they have any unequivocal effect upon salt metabolism (Sexton, 955; Burden, 1956; Matty and Morris, unpublished). Some evidence that his part of the teleost pituitary may be concerned with osmoregulation comes om the work of Arvy, Fontaine, and Gabe (1954) and Arvy and Gabe (1954), ho observe changes in the amount of neurosecretory material contained in he neurohypophysis upon altering the salinity of the water in which fish are mmersed. From the present work and from the examination of a great umber of other teleost pituitaries it does appear that the amount of neuro- ecretory material is greatly variable between fish of the same species. Thus uch qualitative results as have been obtained from salinity experiments hould be repeated in a more quantitative manner.

It has become evident from our observations that histological cell types ifferentiated by Azan and histochemical cell types do not always correspond n different species. For example, orange G cells are PAS-negative in *Phoxinus* ut some orange G cells are PAS-positive in *Pseudoscarus*, and except for the act that they are performic / Alcian blue negative, they are histochemically dentical to type 1 cyanophils. Also in *Rutilus* although all cyanophils are PAS-positive, some have little or no sulphydryl groupings present in their ytoplasm, whereas other cyanophils have abundant —SH radicles. Again in *erca* it is the small orange G cell that has a high tyrosine content, little or no yrosine being found in the cyanophils, but in *Rutilus* it is a cyanophil which as the greatest tyrosine content. All these observations indicate the difficulty f endeavouring to identify a specific hormone-producing cell in all teleost sh upon tinctorial grounds alone. Furthermore the unresponsiveness of the hromophobes and amphiphils to histochemical procedures in these fish again raises the question whether they are reserve cells without secretory function and if under certain circumstances they are able to differentiate into obvious secretory cells.

From the work of Pickford and of Wilhelmi (Pickford and Atz, 1957) it is now well established that the teleost pituitary contains a growth-promoting hormone; however, as mentioned previously, there has been no unequivocal demonstration of the somatotroph although it has been suggested that acidophils, as in mammals, are responsible for the production of the growth-hormone. If the assumption is made that the fish and mammalian growth-hormones are chemically similar (Wilhelmi, 1955) and pituitary cells containing such a hormone are PAS-negative and DDD-positive, then from our observations, since acidophils are less DDD-positive than cyanophils (except for the small orange G cells of the median glandular zone of *Perca*), there are no cells in the fish pituitary which are both PAS-negative and DDD-positive. Thus it may be that the growth hormone is produced in cyanophils along with other glycoprotein hormones. This problem may be answered, along with a recognition of the site of production of ACTH, intermedin, and possibly LH, when histochemical and experimental investigations of the fish pituitary are made together.

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The Action of Fixatives on the Unmasking of Lipid

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SUMMARY

. The capacity of various primary (unmixed) fixatives to split lipids from lipo-
tein complexes was investigated.

. The lipoproteins that formed the test-objects of the research were those con-
ed in (*a*) the acroblast of the cricket (*Acheta domesticus*), and (*b*) the endoplasmic
culum ('ergastoplasm') of the acinar cell of the pancreas of the laboratory mouse.
e two test-objects gave essentially the same results.

. Of the 16 compounds tried, cadmium chloride is the most powerful unmasker
ipids. Mercuric chloride is also a powerful unmasker, and gives much better
tion. Chloroplatinic acid and formaldehyde are moderately powerful unmaskers.

INTRODUCTION

N an earlier study (Clayton, 1958), it was shown that certain compounds
such as ethanol, hydroquinone, &c., act as lipid unmasking agents. This
ect was demonstrated when these agents were used on the tissues after
ation in Flemming's fluid; the lipid was subsequently rendered visible by
Sudan black technique.

Because lipoproteins probably take a general part in the structure of mem-
nes, the demonstration of sites of lipoprotein complexes is of interest with
erence both to light- and to electron-microscopy—and particularly relevant
he latter at the present moment, when the fixation of tissue for examination
the electron microscope is still in its experimental stage. Therefore, it was
nsidered worth while to extend the work to cover the unmasking action of
ostances included in one or other of many fixative mixtures.

Sixteen different 'primary' (unmixed) fixatives were tried. Some of them
lts of cadmium, mercury, and zinc) were shown by Ciaccio (1926) to act
unmaskers; some (salts of cadmium and platinum) have been used to split
d from protein in biochemical analysis (Strecker, 1868); several (arsenious
d, salts of cobalt, and uranium) have been used in fixative mixtures intended
the study of the 'Golgi apparatus' (Golgi, 1908; Da Fano, 1920 *a*, *b*;
món y Cajal, 1914).

The two test-objects chosen were the acroblast of the spermatid of the
use-cricket, *Acheta domesticus*, and the basal endoplasmic reticulum or
gastoplasm' of the acinar cell of the pancreas of the laboratory mouse.
ese objects were selected because a certain amount was already known
out the chemistry of both of them (Bradbury and Clayton, 1958; Clayton,
8; Clayton, Deutsch, and Jordan-Luke, 1958).

MATERIALS AND METHODS

All tissues were rapidly removed from the animals, and individual pieces
both testis and pancreas were placed together in a solution of each one of

arterly Journal of Microscopical Science, Vol. 100, part 2, pp. 269–274, June 1959.]

the particular substances selected for testing as potential lipoprotein splitting agents. The concentration of all the fixatives was approximately that commonly employed when they are used in routine microtechnique.

All tissues were fixed for 18 h, washed, and embedded in gelatine. Sections were cut at 10 μ on a freezing microtome. The Sudan black technique (Baker, 1949) was used to render visible the degree, if any, of lipid unmasking. The intensity of the coloration given by Sudan black was listed according to an arbitrary scale (see appendix, p. 273).

As osmium tetroxide gave a deep brown to black coloration that entirely obscured interpretation of the sudanophilia, the tissues so fixed were bleached for 2 h in 3% (approx.) hydrogen peroxide. Moreover, in order to estimate any possible unmasking action given by hydrogen peroxide itself, the effect of this compound was tried out on gelatine-embedded tissue fixed in formaldehyde. The same concentration of hydrogen peroxide and the same period of action as in the previous experiment were used. The sections were subsequently subjected to the Sudan black technique.

The coloration given by Sudan black does not always indicate that lipids are present, for in certain circumstances this colouring agent is able to act as a dye. There are, however, two tests that can be used to establish the validity of a true lysochrome reaction (Baker, 1958, p. 298). The first is for 'alcohol lability' of the sudanophilia: immersion of the tissue, previously treated by Sudan black, in 90% ethanol for 15 min. The second test consists in the use of Sudan black after pyridine extraction of the tissue for 18 h at 60° C.

A list of the fixatives employed, details of all techniques used in connexion with them, and the results obtained, are given in tables 1, 2, and 3 in the appendix at the end of the paper.

RESULTS

If the sudanophil reaction given by the endoplasmic reticulum of the acinar cell of the mouse pancreas is considered (table 1), it can be seen that the salient facts are as follows.

The strongest splitting reaction of all is given by cadmium chloride; however, the fixation given by this compound is very bad indeed. Mercuric chloride is a rather less powerful unmasker but a much better fixative. Chloroplatinic acid and formaldehyde both give a moderate reaction. Compounds which split lipoproteins, but which at the same time no longer hold the lipid in its original site, are ethanol and chromium trioxide: the lipid is quite often seen in the cell in myelin-like forms.

After the two lysochrome tests (table 2), the nuclei of the acinar cells of pancreas fixed in mercuric chloride no longer exhibit sudanophilia (see p. 274).

The sudanophil responses of the acroblast externum of the cricket spermatid to the action of the primary fixatives are generally so similar to those obtained for the acinar endoplasmic reticulum, that no special discussion is made about them. However, in ethanol-fixed tissues, it can be seen that the id content in the cytoplasm of cells in the pre-division stages of meiosis higher than that in the cytoplasm of cells not at these stages.

Tissue fixed in osmium tetroxide (table 3) gives results somewhat at variance h those given by other fixatives: the endoplasmic reticulum shows no danophilia but other structures such as the acroblast externum, the nucleus, d the zymogen granules are sudanophil.

Discussion

Now that cytological techniques have been used for a hundred years or ore, it seems curious that our knowledge of certain aspects of these tech- ques still tends to be so vague. For instance, so far apparently no one has empted to evaluate the unmasking action, on lipoproteins, of individual ative components. This histochemical omission needs rectifying when it is nsidered that, biochemically, it is now known that lipoprotein, or possibly polysaccharide complex thereof, forms almost the only constituent of the oluble residue which remains after cells have been extracted with salt utions; that is, of the so-called 'ellipsin' fraction of Bensley and Hoerr 934) (Bruemmer and Thomas, 1957; Smith and others, 1957).

Of the primary fixatives used in this study, mercuric chloride in a saturated ution can be recommended both as a rather powerful unmasking agent and a fairly good fixative. The addition of an indifferent salt to the solution of e primary fixative would in many cases undoubtedly improve the general ation of the tissue (Baker, 1958). There is no reason to suppose that the atives mentioned would act less efficiently in splitting lipoprotein complexes used as components of suitable fixative mixtures.

It has been demonstrated by the present study that arsenious acid and salts cadmium and cobalt are all powerful lipid unmaskers. It should also be phasized that these compounds are used as components of fixative mixtures tended for the investigation of the 'Golgi apparatus' (Aoyama, 1929; Da no, 1920 *a*, *b*; Golgi, 1908). Again it should be mentioned that cadmium ts were used both by Strecker (1868) and, with salts of other elements, by accio (1926) in his classical study of lipid unmasking. The latter worker o said that both mercury and zinc might act as unmaskers. It might not be t of place to suggest that the efficacy of some fixative mixtures intended for e study of the 'Golgi apparatus' may largely depend upon the unmasking pacities of the compounds of those elements mentioned above. In fact, this ggestion was foreshadowed by Ciaccio (1926) who, himself, considered that e 'Golgi apparatus' contained a lipoprotein complex.

The two tests, carried out in order to establish whether the sudanophilia own by the nucleus of the acinar cell of the pancreas after mercuric fixa- on was given by lipid, indicate that this was a true lysochrome reaction.

The general results given by osmium fixation suggest that this substance es not act as a lipoprotein splitting agent.

Concerning the high lipid content in the cytoplasm of cells in the pre- vision stages of meiosis, I have noticed that the cytoplasm of the same cells also exhibits a high content of pyronin-positive material, presumably RNA, when they are subjected to the pyronin methyl-green technique (Jordan and Baker, 1955). This present study and that by Bradbury and Clayton (1958) both show that the endoplasmic reticulum of the acinar cell of the pancreas also has a high lipid content. This region likewise has a high content of pyronin-positive material (Lavarack, 1955).

Bradbury and Clayton (1958) suggested that the lipoproteins present in the acinar cell are involved in the composition of the endoplasmic reticulum. Similarly, it may again be suggested here that the cytoplasm in the meiotic testicular cells showing both a high content of lipid and pyronin-positive material may also be associated with the presence of an endoplasmic reticulum.

The writer particularly wishes to thank Dr. J. R. Baker, F.R.S., for much helpful advice and for suggesting the subject of this paper, and also Professor A. C. Hardy, F.R.S., in whose Department the work was carried out.

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APPENDIX

TABLE 1

A summary of the effects of primary fixatives on lipid unmasking

Practical notes			*Results*		
			Cricket	*Mouse pancreas*	
Fixative	*Conc.*	*Sudan black technique*	*Acroblast externum*	*Endoplasmic reticulum*	*Nucleus*
c acid	5%	2½ m	+ to ++ (s.d.)	+ (M)	O
		10 m, 60° C	++ (s.d.)	+ to ++ (M)	O
ious acid	1%	2½ m	(tissue not used)	++ to ++++	O
		10 m, 60° C	—	++ to ++++ (M)	O
hium	1%	2½ m	—	+ to +++	O
oride		10 m, 60° C	—	++++ to +++++	O
roplatinic d	0·75%	2½ m	++ to +++	++ to +++	+
mium	0·5%	2½ m	++ to +++ (M)	++	O
oxide		10 m, 60° C	+++ (M)	++ to +++	+
lt chloride	1%	2½ m	—	+	O
		10 m, 60° C	+ to ++	+++ to ++++	O
er acetate	3%	2½ m	(tissue not used)	+	O
		10 m, 60° C	—	+ to +++	+
hol	100%	2½ m	+++ (M) (E)	+	O
		10 m, 60° C	+++ (M) (E)	+	O
haldehyde	4%	2½ m	—	+ to +++	O
		10 m, 60° C	+++ (d) (M)	++ to +++	O
ochloric	0·1%	2½ m	O to + (?)	+	O
d		10 m, 60° C	O to + (?)	O	O
uric	Sat. soln.	2½ m	++ to +++	++ to +++	O
loride		10 m, 60° C	++++	++++	+++
ium	1%	2½ m	—	O	
roxide		10 m, 60° C	+++	O	++
c acid	Sat. soln.	2½ m	O to +	+	O
		10 m, 60° C	+ (M)	+	O
ssium	1·5%	2½ m	+ (d)	++++	O
chromate		10 m, 60° C	+ to ++ (d)	+++ to ++++	O
yl nitrate	1%	2½ m	—	—	—
		10 m, 60° C	O to + (?)	O	O
sulphate	4%	2½ m	—	++ to ++	O
		10 m, 60° C	+ to +++ (d) (M)	++++ to +++++	O

EY TO TABLE 1: (d) = distorted; (E) = the cytoplasm of cells in the pre-division stages of meiosis bits a lipid content higher than that of the cytoplasm in cells not at these stages; m = minutes; = myelin-like forms present; (s.d.) = slightly distorted; +++++ = very strong reaction; ++ = strong reaction; +++ = moderate reaction; ++ = weak reaction; + = very weak ion; O = negative; — = no observation made.

TABLE 2

A summary of tests carried out on tissue fixed in mercuric chloride in order to establish the validity of the lysochrome reaction of Sudan black

Practical notes			*Results*			
			Tissue			
			Cricket	*Mouse pancreas*		
Treatment before Sudan black	*Sudan black technique*	*Treatment after Sudan black*	*Acroblast externum*	*E.R.*	*Nucleus*	*Z.G.*
none	10 m, 60° C	90% ethanol for 15 m	O	O	O	O
Pyridine extraction for 18 h at 60° C	10 m, 60° C	none	+ to ++	++	O	O

KEY TO TABLE 2: E.R. = endoplasmic reticulum; h = hours; m = minutes; Z.G. = zymoge granules; ++ = weak reaction; + = very weak reaction; O = no reaction.

TABLE 3

A summary of the tests carried out on tissue fixed in osmium tetroxide

Practical notes			*Results*		
			Tissue		
			Cricket testis	*Mouse pancreas*	
Treatment before Sudan black	*Treatment after Sudan black*	*Sudan black technique*	*Acroblast externum*	*Zymogen granules*	*Nucleus*
none	none	10 m, 60° C	+++	++	++
H_2O_2, 3%, for 2 h; pyridine for 15 h at 60° C	none	10 m, 60° C	O	+	+
H_2O_2, 3%, for 2 h	70% ethanol for ½ h	10 m, 60° C	?	++	+ to ++
H_2O_2, 3%, for 2 h	none	none	O	O	O

KEY TO TABLE 3: h = hours; m = minutes; +++ = moderate reaction; ++ = weak reaction; + = very weak reaction; O = no reaction.

An Improved and Rapid Embedding Method

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With one plate (fig. 1)

SUMMARY

lant tissues may be embedded in half the time taken for paraffin embedding if one is used as the dehydrating and infiltrating agent, and the tissue is embedded ster wax. This procedure retains appreciably more lipid material inside the cells does the more usual technique, even when the tissue has been fixed in osmium oxide or in La Cour's 2BE.

INTRODUCTION

WHEN fixed plant tissues are to be sectioned, they are usually dehydrated by passing through graded concentrations of alcohol and are then mersed in chloroform to allow infiltration of the cells by paraffin wax. en performed most meticulously, this process may take as long as 9 days; least time we have been able to use is about 3 days. Moreover, this cedure, especially the treatment in mixtures of chloroform and alcohol, kely to remove lipid material from the tissues and so cause distortion. nce to decrease the time spent in embedding and in an attempt to reduce amount of phospholipid extracted during this process, the use of acetone dehydrating agent and as an infiltrating solution has been investigated. er wax, being more readily soluble in acetone than is paraffin wax, has ded satisfactory results and so has been used in this study.

METHOD

Root tips and pieces of leaves of *Vicia faba* were investigated. After fixation n aqueous fixative such as osmium tetroxide solution, and suitable washing, tissue was treated as follows:

50% acetone in water	1 h
70% acetone in water	2 h
90% acetone in water	2 h
absolute acetone	1 h

t was transferred to fresh absolute acetone. Small pieces of ester wax were ed, and it was left overnight in a container which was corked to prevent poration of the acetone.

The tissue, in this solution in the corked container, was placed in an em- iding oven (about 50° C will suffice) and more solid wax was added, the tainer being shaken so that the molten wax dissolved in the acetone. It was for ½ h.

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The cork was then removed to allow the acetone to evaporate. This was completed in about 1½ h.

The tissue was transferred to a bath of fresh molten ester wax for 10 min and then embedded. Sections were cut at 7 μ, but there seems to be no greater limitation on the thickness that can be cut after this procedure than after chloroform has been used. The wax may be removed from the sections by immersion in acetone and, when required, they may then be mounted directly in euparal.

RESULTS

Sections of roots fixed in acetic-alcohol (1:3 by volume) and embedded by the acetone / ester wax method were identical with those which had been prepared by the usual chloroform / paraffin wax procedures. The embedding o roots fixed in La Cour's 2BE solution (Darlington and La Cour, 1947) was better by the former than by the latter technique. Such sections were stained with either the acid haematein (Baker, 1946) or the orange G / aniline blue (La Cour and Chayen, 1958) methods for lipids; those embedded by the acetone / ester wax procedure contained appreciably more stainable matter especially on the chromosomes (fig. 1, C). Indeed, these sections showed a great retention of lipids, and the same localization of these substances, as did sections fixed in Lewitsky's (1931) fluid (equal volumes of 1% chromic acid and 10% formalin) and embedded by the chloroform / paraffin wax process (La Cour and Chayen, 1958). More striking were the results obtained with roots fixed in a 2% aqueous solution of osmium tetroxide (not buffered) for 1 h. The preservation of root-cells fixed in this manner and embedded by the chloroform / paraffin wax method was extremely poor (also see Chayen and Jackson, 1957) and no granules of any kind were found (fig. 1, A). When embedded by the acetone / ester wax procedure, however, an array of osmiophil granules of various shapes and sizes was found, particularly in the elongating cells behind the meristem (fig. 1, B). Cells of the different regions of the root were distinguishable by their cytoplasmic organization and the vacuoles were

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FIG. 1 (plate). A, part of an unstained longitudinal section through a root of the broad bean *Vicia faba*, which was fixed in a 2% solution of osmium tetroxide and embedded in paraffin wax by the conventional procedure. The cortex and central cylinder are shown; in the cells of neither are cytoplasmic organelles visible.

B, part of an unstained longitudinal section through a root of *V. faba* fixed in 2% solution of osmium tetroxide and embedded in ester wax after dehydration in acetone. Cells of the cortex and central cylinder, from just behind the true meristematic region, are shown; in the latter many osmiophil granules are visible.

C, part of a longitudinal section through a root of *V. faba* which had been fixed in La Cour's 2BE fixative and embedded in ester wax after dehydration in acetone. The section was stained by a modified acid haematein test (La Cour and Chayen, 1958). The embedding is as good a by the conventional procedure but the retention of staining matter, particularly in nucleoli and mitotic chromosomes, is better by this method.

D, transverse section through a leaf of *V. faba*, which had been fixed in Lewitsky's fluid, dehydrated in acetone, and embedded in ester wax. Photographed by phase contrast microscopy. The air space below the guard cells has not collapsed and the chloroplasts are large and distinct.

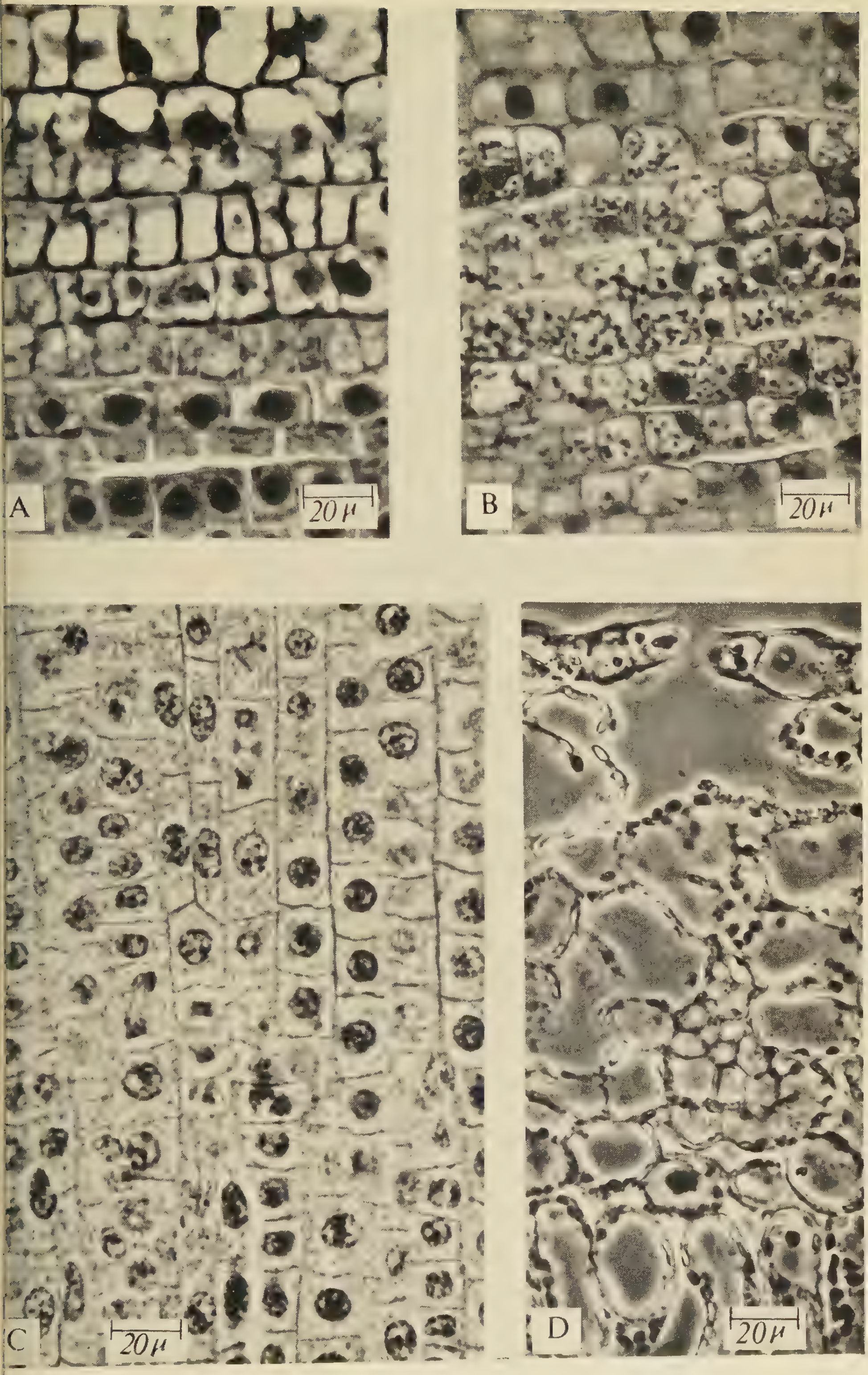

Fig. 1

J. CHAYEN *and* P. B. GAHAN

l delimited. The results obtained by this method of fixation, dehydration, embedding were comparable with those described by Guillermond (1941). e retention of the osmiophil granules or droplets by the acetone / ester wax thod but not by the alcohol / chloroform / paraffin wax technique might icate that the former procedure does retain more lipid material.

The results obtained by both embedding methods with pieces of leaves of *ia faba*, fixed under reduced pressure in 2% osmium tetroxide for 1 h or Lewitsky's fluid for 6 h, were rather similar (see fig. 1, D). The chloroplasts r treatment with alcohol and chloroform seemed to be more shrunken and ssed against the cell-walls than they were by the acetone method. More-r, the birefringence of the cell-walls of the outer layers of parenchymatous s was preserved only by fixation in Lewitsky's fluid and embedding by the tone / ester wax procedure.

DISCUSSION

The results obtained by staining for lipid after fixation in 2BE and the urrence of considerable osmiophil organization after osmium fixation and bedding by the acetone / ester wax method, suggest that this embedding cedure removes less lipid than does the usual chloroform / paraffin wax hnique. Furthermore, although these tissues were embedded more rapidly this process than by that in which chloroform is utilized ($1\frac{1}{2}$ against 3 days), morphological preservation was rather better.

The granules preserved by fixation in osmium tetroxide and embedding by acetone / ester wax procedure deserve further study. All that need be noted present is that they would seem at least to represent osmiophil matter which preserved by this embedding technique but not by that involving the use alcohol and chloroform. It is possible that this embedding method may also of use in the histochemical study of enzymes because of the use of acetone d of the lower temperature of embedding which is theoretically possible by s procedure, especially if purer ester waxes are used (see Chesterman and ach, 1956).

We are grateful to Professor J. T. Randall, F.R.S., in whose Laboratory s method was first designed, and to Dr. H. B. Fell, F.R.S., for her kind vice. We are indebted to Mr. A. L. E. Barron for the photomicrography.

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The Nature of a Chromosomal Phospholipid

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Hertford, Herts.)

SUMMARY

It has been found that the heterochromatic region of interphase nuclei of plant roots d calf thymus, fixed in formaldehyde solution, do not stain for phospholipid as y do after Lewitsky's fixative. It has been shown that this is probably due to the raction of a protein-phospholipid complex. Some lipid-like material has been rified from the used formalin and shown to contain glycerophosphate. The nature the association between the lipid and protein is discussed.

INTRODUCTION

WHEN plant roots were fixed in Lewitsky's (1931) fluid and embedded by the normal procedures, the mitotic chromosomes and the hetero- romatic regions of the interphase nuclei gave apparently positive reactions phospholipids by the acid haematein test and stained yellow in a mixture orange G and aniline blue (La Cour, Chayen, and Gahan, 1958; compare ker, 1946). If, however, the tissues had been fixed first in formaldehyde- cium (Baker, 1946) and then in Lewitsky's solution, neither the mitotic romosomes nor the interphase heterochromatic regions reacted with the d haematein and they stained blue in the double stain. On the other hand, en such tissues were treated with hot pyridine, hot ethanol-ether, or with % trichloroacetic acid at 90° C for 15 min, the whole of the interphase and totic chromosomes gave an intense reaction with the acid haematein method d stained yellow in the dye mixture. It might have been thought that the fference in staining produced by prefixation in formaldehyde-calcium dicated that this solution had removed a chromosomal phospholipid. The jection to this conclusion was that, after such fixation, methods which were lieved to remove lipids produced enhanced, not reduced, staining, so sting doubt on the validity of the histochemical reactions for phospholipids. he problem of the enhanced staining formed the basis of a separate investi- tion (see Chayen, Gahan, and La Cour, 1959). The question whether the fference in staining behaviour after the two fixations could have been due some solvent action of the formaldehyde-calcium forms the subject of the esent study.

We have included some results obtained with calf thymus. In this tissue the clei, when fixed in Lewitsky's fluid, stain uniformly yellow in the double- ain and react positively to acid haematein. As in roots, these reactions are hibited by formaldehyde-calcium fixation.

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MATERIAL AND METHODS

Material. The terminal 5–10 mm of roots of *Trillium grandiflorum* grown i soil in pots and of roots of *Vicia faba* grown in damp filter paper were used i this investigation. Calf thymus was also studied: the gland was removed from the animal immediately after slaughter and packed in dry ice for transportation to the laboratory.

Methods. The technique for purifying and hydrolysing phospholipids and for their chromatographic analysis are described fully in another communication (Chayen, Gahan, and La Cour, 1959). The staining methods were the modified acid haematein and the orange G aniline blue procedures used by La Cour and others (1958), as well as the usual alcoholic Sudan black method The fast green technique of Alfert and Geschwind (1953) was also studied deamination was performed by leaving sections at room temperature for 10 min in a solution composed of equal volumes of 0·1 N hydrochloric acid and 0·1 N sodium nitrite, which were mixed immediately before use.

RESULTS

The materials to be studied, namely 200 root tips of *T. grandiflorum*, 1500 of *V. faba*, and small pieces of calf thymus, were fixed for 6 h in formaldehyde-calcium and were then transferred to fresh fixative for a further 24–48 h Some roots were studied cytologically to see whether the mitotic and interphase chromosomes gave any reaction for phospholipids. The used fixatives were centrifuged to remove solid matter and, if not completely clear, they were filtered under reduced pressure through a Seitz filter. When completely clarified, their ultraviolet absorption was examined in a Beckman spectrophotometer and they were then shaken with equal volumes of chloroform A heavy milky precipitate formed which, on standing, came to rest at the interface between the chloroform and the fixative. This precipitate was removed together with the chloroform and the fixative was again shaken with fresh chloroform, this procedure being repeated until almost all the precipitable material had been removed. The ultraviolet absorption of the residual formaldehyde-calcium was tested. Since the behaviour of the matter extracted by the long fixation was the same as that by the short treatment, the two precipitates usually were combined.

It was then necessary to dissolve in methanol-chloroform (1:2, v/v) any phospholipid present in the precipitate. In the large-scale experiments with calf thymus most of the chloroform was drained off first; with the plant matter, which was obtained in small quantities only, the chloroform was retained and a suitable volume of methanol was added to it. The precipitate was shaken gently with a large volume of methanol-chloroform and was allowed to stand at room temperature for about 1 week. During this time, much of it dissolved, leaving a white flaky or fluffy residue which had to be separated from the clear solution. The absorption characteristics of the clear solution were examined. The fluffy residue obtained from calf thymus was hydrolysed with 6 N hydrochloric acid for 5 h and on chromatographic analysis was found contain a large number of amino-acids. These were difficult to resolve by methods used, but spots having the R_F value of arginine, serine, and ssibly ethanolamine were seen. Free phosphate, and some material which cted like choline but which did not run free were also detected. These ults would indicate that this residue which was insoluble in methanol-oroform might consist of a protein which was associated with a lipid-like stance, possibly of a type resembling sphingomyelin.

The solution in methanol-chloroform was subjected to the procedure of lch (see Chayen, Gahan, and La Cour, 1959). As the methanol escaped into water the solution containing the suspected lipid became milky and finally, en all the methanol had diffused into the water, a white milky precipitate med at the interface between the chloroform and water. The chloroform l the precipitate were redissolved in methanol-chloroform and the pro-dure was repeated with the same results.

A suitable volume of methanol was again added to the chloroform to solve the precipitate and the absorption spectrum of the solution was amined. The solvent was evaporated off; some of the residue was examined croscopically, while the rest was hydrolysed in alcoholic potash and estigated by partition chromatography. No choline, serine, ethanolamine, or sitol were found, although slight traces of other amino-acids were seen asionally. Glycerophosphate was identified repeatedly. In other tests (by method of McKibbin and Taylor, 1949) no sphingosine could be detected.

Histochemical tests were performed on the precipitates. The matter which s precipitated out of the fixative by shaking it with chloroform gave strong ctions for arginine by the method of McLeish and others (1957); both this terial and the purified lipid-like matter yielded positive reactions with the d haematein and the orange G aniline blue methods. When the lipid-like terial was dried from solution in methanol-chloroform it formed an almost stalline pattern which altered drastically on wetting.

When root tips, or calf thymus, were fixed in Lewitsky's fluid and the used ative shaken with chloroform, no precipitate was formed. Similarly, no ecipitate was obtained from the used fixative when a methanolic solution of monium reineckate was added to it; this reagent produced a heavy precipi-e when added to formaldehyde-calcium which had been used to fix plant ots.

Since the material extracted by formaldehyde-calcium yielded a strong ction for arginine, it was advisable to study the effect of this fixative histo-emically. Sections of roots of *T. grandiflorum* and of *V. faba* which had en fixed in either formaldehyde-calcium or in Lewitsky's fluid were tested th the modified arginine reaction (McLeish and others, 1957). The nuclei those fixed in Lewitsky's solution stained more intensely, especially in the terochromatic regions, than did those treated with formaldehyde-calcium.

An attempt was made, therefore, to use the fast green method, which is d to be specific for basic proteins of the histone type (Alfert and Gesch-nd, 1953). The dye was dissolved at about pH 8, the pH being adjusted either by the addition of alkali or by adding the dye to a citrate-phospha buffer solution at this pH. The nuclei in sections of roots which had bee fixed in the chromic fixative stained very much more intensely than did tho which had been treated with formaldehyde-calcium. Staining after eith fixation was stronger with the buffered than the unbuffered solution, and w largely removed if the tissue was first deaminated.

Discussion

The material removed by formaldehyde-calcium. It is clear from the absor tion curves in Chayen, Gahan, and La Cour (1959) that formaldehyd calcium removes from calf thymus and from *Trillium* roots some materi which resembles nucleic acid or nucleotides (peak about 260 mμ) and prote (peak about 280 mμ). After it has been shaken with chloroform, almost all th nucleotide-like matter is left in the fixative but much of the substanc absorbing maximally at about 280 mμ has been precipitated out of solutio This precipitate stained with the arginine reaction; similarly after this fix tion, the interphase nuclei stained less strongly for basic proteins and fo arginine than they did after fixation in a fluid from which no such precipita was obtained.

Histochemical data indicated that formaldehyde-calcium, but not Lewitsky fluid, removed matter particularly from the heterochromatic regions of inte phase nuclei and from mitotic chromosomes. The results with the argini and the fast green methods suggested that formaldehyde-calcium removed protein. The loss of staining with the acid haematein and the orange aniline blue procedures after this fixative were less certain indications of th simultaneous removal of a phospholipid from the same sites owing to doub concerning the validity of the techniques. It has now been shown that from th used formaldehyde-calcium, but not from used Lewitsky's solution, matte can be precipitated which may be resolved into two components, dependin on their solubility in methanol-chloroform. The insoluble material, on hydro lysis, yielded chromatograms which confirmed the view that formaldehyd calcium removed protein; there was some indication that it was close associated with some lipid-like substance resembling sphingomyelin. Th soluble matter seemed to contain traces of substances resembling nucleotid (curve (iii), fig. 1, B, in Chayen, Gahan, and La Cour, 1959). These must hav been intimately associated with lipid in that they were soluble in methano chloroform but they were largely removed by the prolonged Folch procedur The material that remained confirmed the histochemical results for phospho lipid in that it yielded glycerophosphate after hydrolysis, but it was excep tional in that no choline, serine, ethanolamine, or reducing sugars wer detected. It is unlikely to be free glycerophosphate owing to the nature of i isolation. Hence, despite the lack of proof for the presence of fatty acids, must be considered tentatively to be either a phosphatidic acid or a phospho lipid which resembles lecithin in containing glycerophosphate but in whic the choline is replaced by an unknown nitrogen base. Determination of th atio of phosphate to nitrogen did not clarify the problem since varying mounts of protein or peptide compounds were left after the Folch procedure, s is observed frequently when true lipo-proteins are subjected to this treatment.

The nature of the lipid and protein. It seems possible, therefore, that as was uggested by the histochemical data, formaldehyde-calcium removes from alf thymus and from roots of *T. grandiflorum* and *V. faba* a phospholipid nd a protein which may have a high proportion of basic amino-acids. The vidence indicates that they are removed as a complex which is split by the ater treatment. It should be emphasized that, in agreement with Baker (1946), o other phospholipids were extracted by this fixative as far as could be etected. The problem, therefore, is why this phospholipid should be the xception and why formaldehyde has extracted a protein.

It may not be unreasonable to suggest that this problem could be resolved f the phospholipid and protein were associated in such a way that the lipid rotected the free amino-groups of the protein against fixation by formalin hile the protein rendered the complex soluble in an aqueous medium. This might be achieved, for example, if a phosphatidic acid were esterified with uch groups as the guanido-amino of arginine. It is noteworthy that Barnard nd Danielli (1956) found that histidine which was present in nuclei, and redominantly in the heterochromatic regions (Barnard, personal communicaon), was protected against benzoylation by combination with an unknown ubstance which was thought to be deoxyribonucleic acid. It seems possible hat their results might have been caused by the combination of the aminocid with the lipid-like substance reported in the present communication.

The evidence that the complex is present on chromosomes. When plant roots r mouse liver are fixed in Lewitsky's fluid, the heterochromatic regions of nterphase nuclei and the mitotic chromosomes stain for phospholipid but hey fail to do so when fixed in formaldehyde-calcium (see La Cour, Chayen, nd Gahan, 1958; Chayen, Gahan, and Harvey, in preparation). A lipidrotein complex has been isolated from the latter, but not from the former xative after use, so confirming the gross histochemical findings. Two quesions remain to be answered; the first concerns the reliability of the staining rocedures, and the second the possibility of a diffusion artifact in the histohemical localization of the lipid.

(i) The validity of the staining methods has been examined and discussed lsewhere (Chayen, Gahan, and La Cour, 1959; La Cour and others (1958). Moreover the same distribution of lipids has been observed when benzpyrene, fluorochrome, has been used after these fixatives (Chayen and others, 1957). While it might be argued that the acid haematein method is staining some nterfering substance, there can be no doubt that benzpyrene concentrates only n lipid material (Berg, 1951).

(ii) When fixation was effected by formaldehyde-calcium containing ammonium reineckate, a precipitant of this lipid-protein complex, the distribuion of phospholipid was identical with that found after Lewitsky's fixation.

On the other hand, obvious diffusion and precipitation artifacts were observe when this salt was added to methanol that was used for fixation (La Cour an others, 1958). In addition, benzpyrene has been localized in heterochromat regions of interphase nuclei of living macrophages from mouse lung grown tissue culture, which suggests that these regions contain lipid in life (Chaye Gahan, and Harvey, in preparation).

We wish to thank Professor J. T. Randall, F.R.S., for his encourageme and for laboratory facilities. We are very grateful to Dr. H. B. Fell, F.R.S and Dr. C. Long for helpful advice.

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The Histology of the Nervous System of an Insect, *Rhodnius prolixus* (Hemiptera)

I. The Peripheral Nervous System

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With one plate (fig. 6)

SUMMARY

The histology of the peripheral nervous system of *Rhodnius* has been re-investigated by the use of the osmium / ethyl gallate method of staining. The fusiform enlargements (beading) of the fine sensory axons, both below the epidermis and in the larger nerves, mark the points at which a mitochondrion lies in the axoplasm. The large motor axons contain many mitochondria; sometimes these form chains as much as 20 μ long.

The formation of the interaxonal substance in the nerves by the glial cells (Schwann cells) is well shown by this staining method.

The cytoplasmic component of the nerve-sheath (perineurium) contains abundant mitochondria; this layer, which is rich in enzymes, seems largely responsible for the permeability properties of the sheath. The connective tissue component (neural lamella) contains collagen.

In the thin-walled sensilla of the antennae (presumed chemoreceptors) the distal nerve process ends in a small orifice near the base of the hair (as described by Slifer in grasshoppers). The axons from the sense cells in the antenna fuse extensively; at least 15 sense cells to an axon. This does not happen in the sensory axons from the leg.

The nerve-endings in the ventral abdominal muscles are described and figured.

WHILE studying the osmium/ethyl gallate method of fixation and staining (Wigglesworth, 1957) a number of incidental observations were made on the nervous system of *Rhodnius*. This staining method reveals particularly those structures most rich in lipids, the cell membranes, fat droplets, mitochondria, and 'Golgi element', giving a picture which closely resembles that seen with the electron microscope. This appearance is so different from that shown by standard methods of staining, that it seemed worth while to re-investigate the histology of the nervous system of the insect by its use. That should provide a useful background to the electron microscope studies being made at the present time. In this paper, the first of three which are projected, certain parts of the peripheral nervous system are described.

METHODS

Many of the observations have been made on whole mounts. The abdominal walls of 4th- or 5th-stage larvae of *Rhodnius prolixus* Stål have been fixed in buffered osmium tetroxide (Palade, 1952) for 1 to 2 h, rinsed in Ringer's solution, transferred to the saturated solution of ethyl gallate in

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0·25% cresol for 24 h, and then mounted in Farrants's medium containing ethyl gallate.

Material for sectioning has been fixed in the same way for 4 h, stored for 24 h in the ethyl gallate solution, and embedded in ester wax (Steedman, 1947). Sections have been cut at 0·5 to 2 μ and again mounted in Farrants's medium with ethyl gallate.

SENSE CELLS AND AXONS OF THE ABDOMINAL WALL

The abdomen of the *Rhodnius* larva bears numerous spatulate hairs each arising from a smooth rounded plaque. Each hair is innervated by a single sense cell and axon (Wigglesworth, 1953). In addition there is a smaller number of campaniform organs similarly innervated.

Fig. 1, A shows the innervation of one of these sensory hairs after staining with methylene blue by Dogiel's method. The distal process, the sense cell and the axon are stained. The axon often shows small fusiform dilatations (marked by arrows).

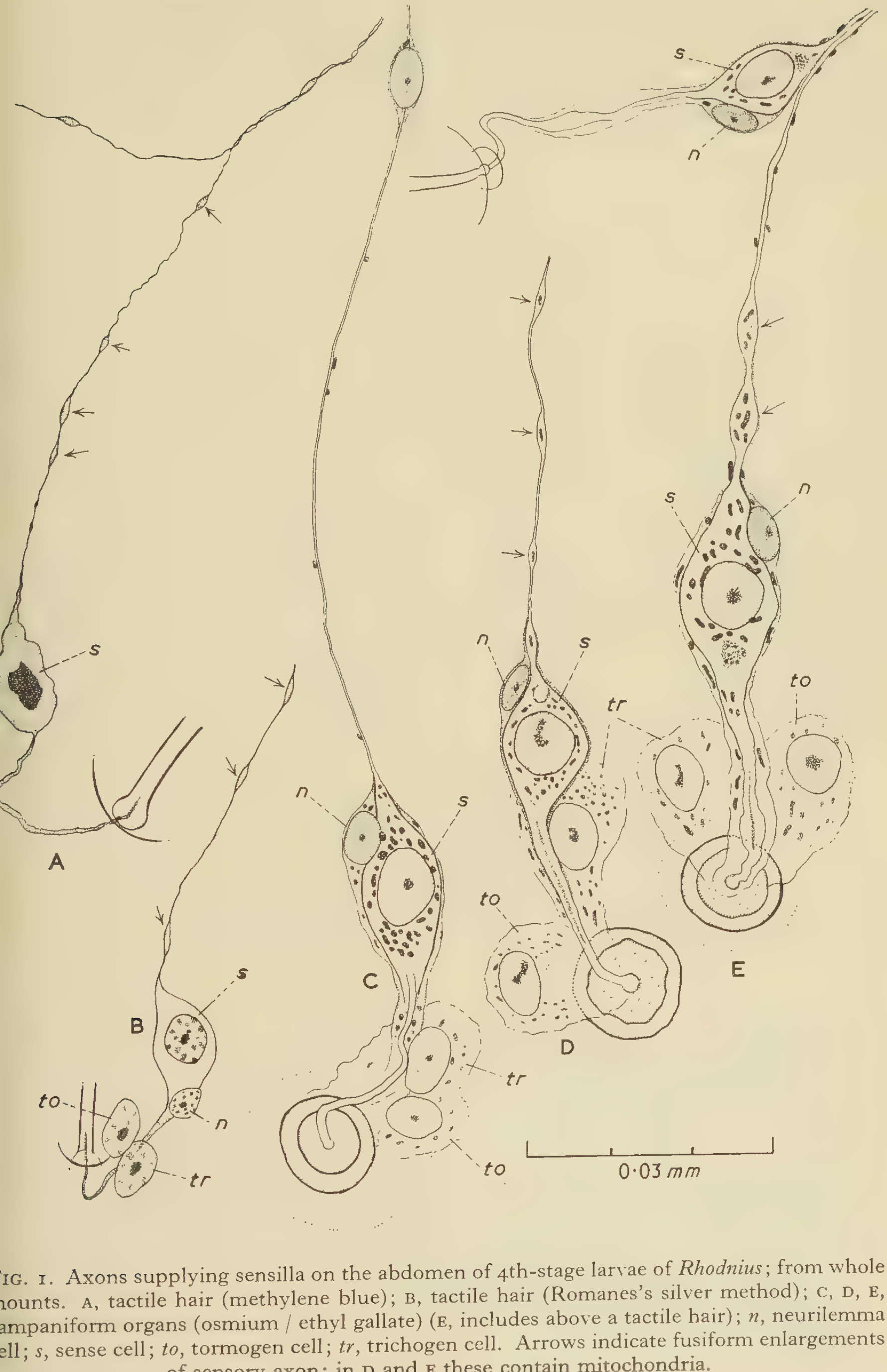
Fig. 1, B shows the corresponding structures stained with the silver method of Romanes after fixation in Bouin's mixture. The outline of the sense cell is clearly visible and the nuclei of the trichogen, tormogen, and neurilemma cells can be seen. The axon again shows small fusiform enlargements of the same general appearance as after methylene blue staining.

Fig. 1, C–E shows the innervation of campaniform organs after staining with the osmium/ethyl gallate method. (The sense cells supplying the campaniform organs are larger than those supplying the hair sensilla; in fig. 1, E the axon from the campaniform organ is later joined by the axon from one of the smaller sense cells of a hair sensillum.) The position of the trichogen and tormogen cells is lightly indicated; the other parts are carefully drawn, the background of epidermal cells being omitted for the sake of clearness.

The sense cells contain conspicuous mitochondria, but the axons are so fine that there is no room for mitochondria in the lumen and, as seen in fig. 1, C, they may run long distances without any contained mitochondria. In other axons the same fusiform enlargements as observed in fig. 1, A, B can be seen, and each of these dilatations marks the position of a mitochondrion within the axoplasm (fig. 1, D).

Fig. 1, E was taken from an insect in the early stages of moulting, and shows conspicuous dilatations of this kind, some of them containing several mitochondria. In addition to the mitochondria, the sense cells may contain one or more vacuoles, and sometimes a diffusely staining inclusion containing strongly osmiophil granules. Perhaps this represents the Golgi element.

The neurilemma cell is closely applied to the surface of the sense cell. The nucleus is small and the homogeneous nuclear sap more deeply staining than that of the other nuclei. The cytoplasm of the neurilemma cell is more deeply staining still and forms a sheath for the sense cell; this sheath extends along the distal process and along the axon. The cytoplasm of the neurilemma cell also contains mitochondria, and the mitochondria which can often be seen



IG. 1. Axons supplying sensilla on the abdomen of 4th-stage larvae of *Rhodnius*; from whole ounts. A, tactile hair (methylene blue); B, tactile hair (Romanes's silver method); C, D, E, ampaniform organs (osmium / ethyl gallate) (E, includes above a tactile hair); *n*, neurilemma ell; *s*, sense cell; *to*, tormogen cell; *tr*, trichogen cell. Arrows indicate fusiform enlargements of sensory axon; in D and E these contain mitochondria.

applied to the surface of the axon (e.g. in fig. 1, C, E) are probably mitc chondria of the neurilemma cell. Similar cells, of unknown origin (Wiggle worth, 1953), occur along the axons and smaller nerves (e.g. in fig. 1, C).

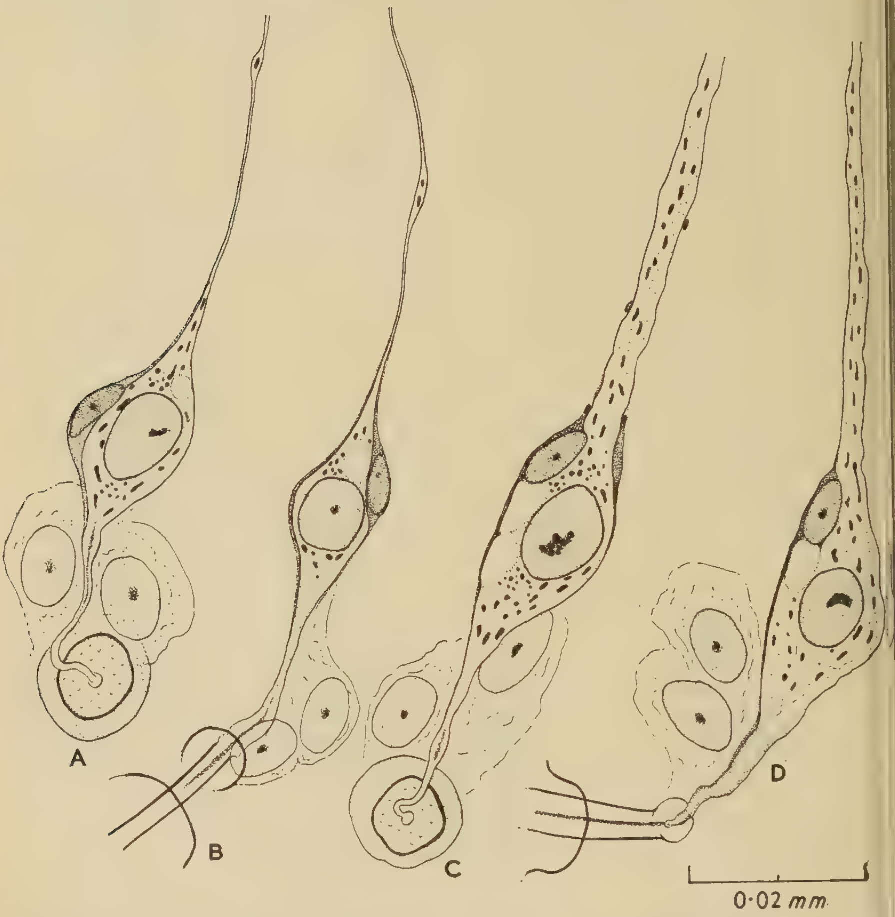


FIG. 2. Sense cells and axons supplying sensilla on the abdomen of 4th-stage larva of *Rhod nius*; from whole mounts. A, campaniform organ on uninjured side; B, tactile hair on un injured side. C, campaniform organ (corresponding in position to A) 4 days after axon wa interrupted by burning; D, tactile hair, 4 days after burning of axon, for comparison with B

The appearance seen in fig. 1, E suggests that mitochondria produced i the body of the sense cell may be passed down along the axon. In the earlie work (Wigglesworth, 1953) it was shown that if the axon is destroyed b burning, the sense cell becomes highly active and the axon regenerates an grows inwards to the central nervous system. It was to be expected that th movement of mitochondria into the axon would be most evident under thes conditions.

Fig. 2 illustrates experiments of this type. Fig. 2, A, B shows the nerve supply of a hair sensillum and a campaniform organ on the normal untreated side of the abdomen. Fig. 2, C, D shows corresponding sensilla on the opposite side of the abdomen, where the axons had been destroyed by burning 4 days earlier. On the normal side the appearance is as already described. In the sensilla with regenerating axons, the sense cells are somewhat enlarged; the nucleoli in the sense cells are greatly enlarged; and mitochondria are entering the thickened axons in large numbers.

SENSILLA ON THE ANTENNAE

In an earlier paper (Wigglesworth and Gillett, 1934) it was shown that there are 4 types of sensillum on the antenna of *Rhodnius*: (i) large tactile hairs which project well beyond the innumerable small sensilla; (ii) small thin-walled trichoid sensilla each supplied by a large fusiform group of about 15 sense cells. These were regarded as olfactory organs; (iii) slender, relatively thick-walled sensilla with 5 or 6 sense cells. This is the most abundant type; they were considered to be probably thermal receptors; (iv) very similar to type (iii) except that the hair is much shorter.

The tactile hairs have the same structure as those of the abdomen and elsewhere and need not be described. But it was of interest to re-investigate the structure of type (ii), because Slifer has recently shown that in many of the olfactory trichoid sensilla of grasshoppers the distal nerve process ends, not in the general cavity of the organ, but in a permeable spot on its surface. In some of the organs this lies at the tip of the sensillum (Slifer, Prestage, and Beams, 1957); in others it is close to the base (Slifer, 1956). Sections of the antenna of *Rhodnius* fixed and stained with osmium and ethyl gallate revealed a similar arrangement (fig. 3, A).

Below the insertion of the thin-walled hair there is a clear unstained cavity surrounded by a mantle of deeply staining cytoplasm containing mitochondria. This cavity is occupied by what look like slender tubules (0·2 to 0·3 μ in diameter), which run from the sense cells to the base of the sensillum. There they seem to be connected with a single osmiophil strand that ends in a deeply staining dome-shaped structure which lies in the floor of a minute pit near the base of the thin-walled hair.

The large trichogen cell contains abundant mitochondria and there are mitochondria in the sense cells. The whole structure closely resembles that in the grasshopper as demonstrated by Dr. Slifer in electron microscope sections at the International Congress of Cell Biology held at St. Andrews, Scotland, in 1957.

The cellular parts of types (iii) and (iv) are similar. Fig. 3, B, B′ shows details of one of the small thick-walled sensilla (type (iv)). A group of 6 sense cells give off distal tubular filaments which enter a small bulb-shaped cavity. This cavity is continued as a clear canal which extends right into the sensillum. A single fine darkly staining filament runs through the centre of this canal. The canal and the bulb at its base are enclosed in a sleeve of deeply staining cytoplasm containing numerous mitochondria.

Fig. 3, c shows the connexion between the group of sense cells of a type (ii) sensillum and the antennal nerve. As we have already seen, the axons do not stain with osmium and ethyl gallate, but their course is indicated by the sheaths and by the rows of mitochondria which they contain.

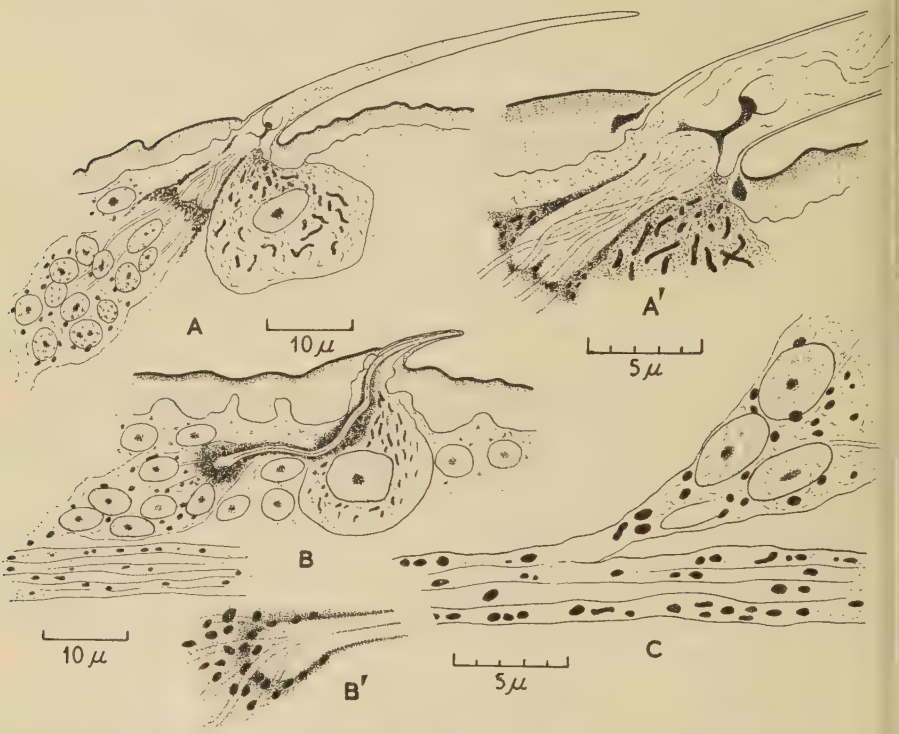


Fig. 3. Longitudinal sections of terminal segment of antenna of *Rhodnius* adult. A, thin-walled sensillum, type (ii); A′, detail of distal processes of the sense cells in the same; B, sensillum type iv; B′, detail of distal processes of sense cells; C, axon processes of sense cells of type (iv) sensillum joining the antennal nerve.

By making a rough count of the numbers and types of sensilla on the antenna of an adult *Rhodnius* it was estimated that in the terminal segment alone there are something like 4,000 to 5,000 sense cells. The number of axons in a transverse section of the antennal nerves is not easy to estimate, for they are not sharply walled off from one another but are in process of fusion. A generous estimate gave a maximum of 150 axons in a single nerve at the base of the terminal segment, say 300 axons in the two nerves. This represents a degree of fusion of at least 15 sense cells to one axon. The true figure is probably greater than this.

In transverse sections of the antennal nerves the boundaries of the axons are somewhat ill-defined; many of them contain mitochondria seen in cross-section. A distinct 'perineurium' and 'neural lamella' are not present but a delicate sheath encloses the nerve. This is doubtless the product of the glial cells (Schwann cells) lying within the nerve or on its surface. The cytoplasm of the Schwann cells radiates between the axons; it contains numerous mitochondria, which are seen in cross-section as dark points between the axons, and probably other material rich in lipids. A number of the mitochondria lie immediately below the nerve-sheath.

In longitudinal sections of the antennal nerves the axons are often seen to have fusiform dilatations. They contain mitochondria of varied size and shape. Fig. 6, A is a 2 μ section photographed so that the axis of most of the large nerve-fibres is in focus. Fig. 6, B shows the same section focused slightly higher so that the more darkly staining interaxonal material is seen. The mitochondria which appeared as dark points in cross-section are now seen as threads oriented in the long axis of the nerve.

NERVES AND NERVE-ENDINGS OF THE VENTRAL ABDOMINAL MUSCLES

The ventral abdominal muscles in *Rhodnius* are fully developed and functional only at the time of moulting. The contractile fibrils then undergo autolysis and only the nuclei and muscle-sheath remain. The fibrils are developed anew before the next moult (Wigglesworth, 1956).

The nerves supplying these muscles are very readily observed in whole mounts, in which their endings can be studied with the highest powers of the microscope. These endings persist apparently unchanged even when the contractile fibrils have disappeared (Wigglesworth, 1956). This is therefore very suitable material on which to observe the motor endings when stained by the osmium / ethyl gallate method.

Fig. 4, A shows the nerve-supply to a single muscle. The nerve runs transversely across the middle of the muscle giving off branches which spread over the surface or along the margins. We shall see that these two parts, the transverse nerve and the branches to the muscle, have a totally different structure.

Fig. 4, B is from a methylene blue preparation of the transverse nerve and the beginning of one of the muscle branches. The axons in the main nerve show occasional fusiform swellings, and the branches to the muscle are seen to be exceedingly fine. No other structural details are revealed.

Fig. 4, C shows a similar region fixed and stained with osmium / ethyl gallate. The transverse nerve contains only occasional glial nuclei ('lemnoblasts' of Edwards and others (1958), Schwann cells of Hess (1958)) and is enclosed in a delicate sheath which is often connected to adjacent lobes of the fat body, &c., by connective tissue strands formed by the haemocytes (Wigglesworth, 1955). The unstained axons are rendered visible by the staining of the axon sheath. They show frequent fusiform enlargements, each of which usually contains a mitochondrion. There are occasional mitochondria outside the axons, presumably belonging to the cytoplasm of the glial cells.

The only other visible feature of the nerves is a series of flattened or lozenge-shaped inclusions which stain a diffuse grey, somewhat like enormous mitochondria. The nature of these inclusions is not known; they might repay study with the electron microscope. They occur along many of the smaller nerves and

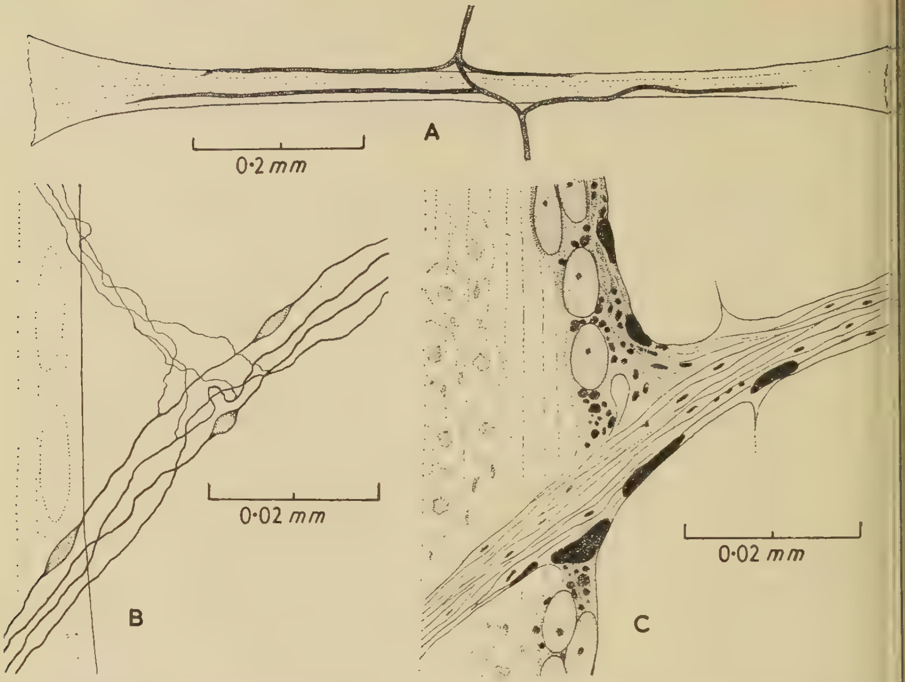


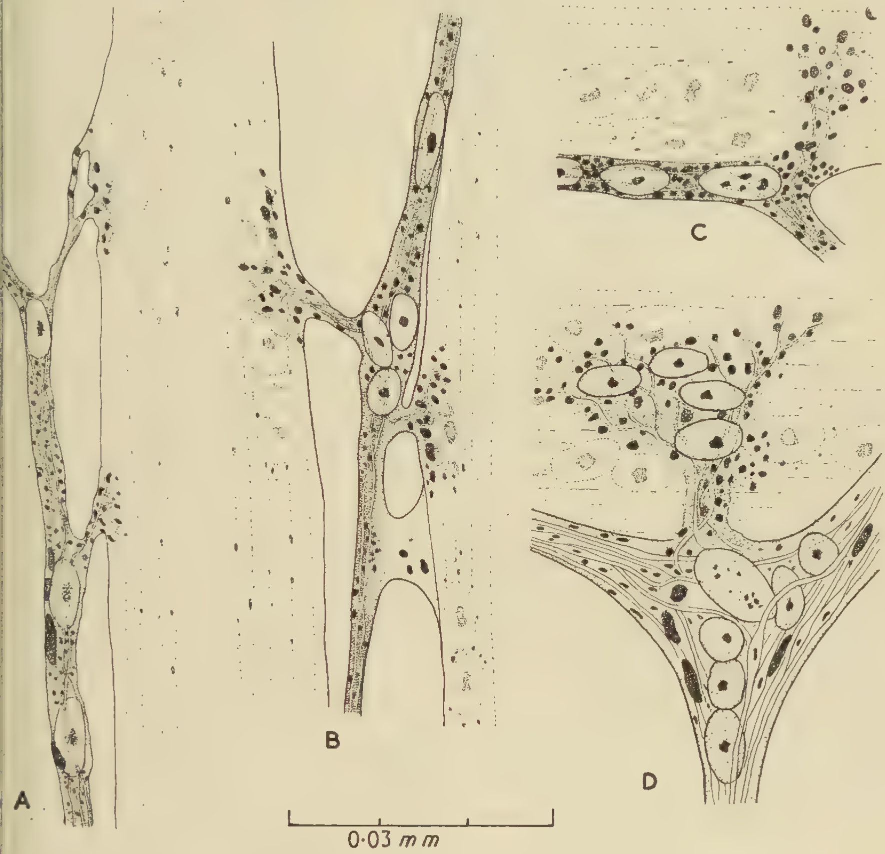
FIG. 4. A, single ventral abdominal muscle from 4th-stage larva of *Rhodnius* showing nerve supply. B, transverse nerve giving off (above) a branch to the muscle (methylene blue). C, transverse nerve giving off branches (above and below) to the muscle (osmium / ethyl gallate). Nuclei of nerve-sheath with solid outline, muscle nuclei with dotted outline. Note mitochondria in the axons, mitochondria in the nerve-sheath, and large, darkly staining elongated inclusions in the nerve-sheath.

sometimes along the branches to the muscle (fig. 5, A). They stain supravitally with Nile blue sulphate (1 : 1,000) and Janus green (1 : 50,000).

The branches to the muscle have a totally different structure. They consist of a thick sleeve of darkly staining cytoplasm, richly supplied with nuclei and packed with mitochondria (figs. 4, C; 5, A–C). The axon filaments can be seen as colourless threads in this dark cytoplasm. As is well known, the connective tissue sheath of these nerve-branches becomes continuous with the sheath of the muscle (Tiegs, 1955; Edwards and others, 1958), so that when the axons send off side branches or terminal branches to end in the muscle, these lie beneath the sarcolemma.

The nerve-endings seem to be quite irregular in their distribution over the surface of the muscle. In these endings also the branching axons are surrounded by cytoplasm which stains with osmium / ethyl gallate and contains abundant mitochondria. These endings may contain nuclei (as in fig. 5, D), or nuclei may be absent (as in fig. 5, A–C). (In the figures the muscle nuclei are faintly indicated in dotted outline to distinguish them from the nuclei of the nerve-endings.)

It is sometimes said that muscle end-plates do not exist in insects (Meyer, 955; Wigglesworth, 1956); but that refers to the elaborate folded memranes rich in cholinesterase which are characteristic of nerve-endings in ammals. End-plates characterized by 'synaptic vesicles' in the nerve-ding, abundant mitochondria, and 'aposynaptic granules' in the muscle bstance have been described by Edwards and others (1958) in the leguscles of the wasp. At this stage there is no purpose in comparing these ructures with the endings studied with the light microscope in *Rhodnius*, art from pointing out that no mitochondria have been seen in the fine rminal branches of the nerves in *Rhodnius*. The end-plates of *Rhodnius* do t contain cholinesterase (Wigglesworth, 1958).



G. 5. Nerve-endings in ventral abdominal muscles, showing fine terminal branches in rkly staining cytoplasm with mitochondria. Nuclei of nerve-sheath with solid outline, muscle nuclei dotted. In D, part of the main transverse nerve is seen below.

STRUCTURE OF LARGER NERVES

The structure of the main nerves of the legs has been studied as they proach the fused ganglia. The nerve of the mesothoracic leg in the 5th-stage larva is about 40 μ in diameter. The larger axons, predominantly motor axon lie chiefly in the dorsal half (fig. 6, C). They number about 130 and range size from 1 μ to 5 μ in diameter. The small sensory axons lie chiefly in t ventral half of the nerve; they number about 2,000; some are as much as 1 in diameter but most are 0·5 μ or less.

A rough count of the sensilla (tactile sensilla and campaniform organs) all segments of the mesothoracic leg gave a total of about 2,100. The numb of small axons in the corresponding nerve was estimated by counting those a small area of the cross-section and multiplying by the total area. This ga a figure of about 1,900. These figures suggest that, unlike the axons in t antennal nerve, the axons from the tactile sensilla of the legs do not fuse wi one another.

Fig. 6, H shows a longitudinal section through the dorsal half of the mes thoracic nerve. The large axons are more or less uniform in diameter f considerable distances. Their contents have a faintly fibrillar appearance, b are otherwise unstained. They contain, however, large numbers of mit chondria of all sizes. These are sometimes in pairs, as though they h recently divided; and occasionally they occur in chains 20 μ in length, co taining as many as 25 mitochondria which diminish progressively in si towards the extremities and appear to be incompletely separated from o another (fig. 6, F, G).

The large nuclei of glial cells (Schwann cells) occur at intervals and t darkly staining cytoplasm from these extends between the axons to form t sheaths by which the axons are separated. This interaxonal cytoplasm al contains mitochondria elongated in the long axis of the nerve and perha other inclusions rich in lipids; occasionally it contains rows of small vacuol (fig. 6, J).

Fig. 6, K shows a longitudinal section through the ventral half of the nerv There is one axon of 3 μ diameter down the middle, but most axons are in t 0·5 to 1 μ range. Throughout the greater part of their length these small axo

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FIG. 6 (plate). A, longitudinal section of antennal nerve at base of subterminal segme cut at 2 μ: mitochondria in the axons.

B, the same at slightly higher focus, showing mitochondria in axon sheaths.

C, transverse section (0·85 μ) of mesothoracic nerve, showing motor axons above and mai sensory axons below. *tr*, tracheae in the perineurium; the arrows indicate nuclei of Schwa cells.

D, tangential section of nerve-sheath where it joins the ganglion, showing filamentous mit chondria.

E, the same showing tracheae (*tr*) in perineurium.

F, longitudinal section of motor axons (2 μ section) showing chains of mitochondria (inc cated by arrows).

G, the same at slightly different focus.

H, longitudinal section (at 2 μ) through motor axons in mesothoracic nerve.

J, the same cut at 0·85 μ. Note two Schwann nuclei (indicated by arrows) with dark staining cytoplasm extending between the axons and containing rows of vacuoles.

K, longitudinal section at 0·85 μ through sensory part of mesothoracic nerve. Arrow inc cates a Schwann cell nucleus with darkly staining cytoplasm extending between the axons.

L, single collagen fibril in neural lamella. Electron microscope section by Dr. D. S. Smit

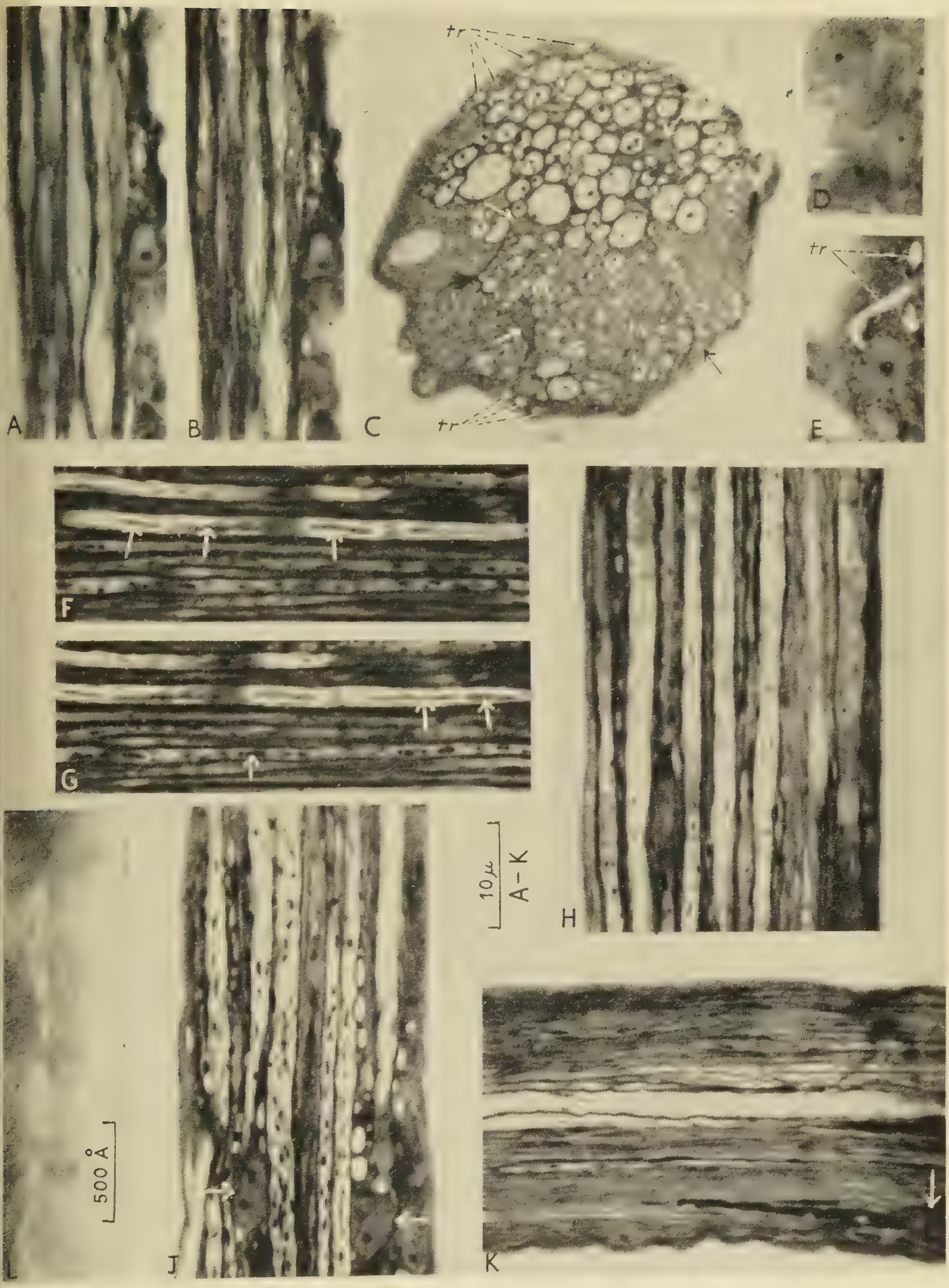

FIG. 6

V. B. WIGGLESWORTH

do not contain mitochondria, but from time to time they show fusiform swellings with a mitochondrion within them.

The existence of these swellings has been confirmed by examining thick (4 μ) transverse sections. On focusing up and down it is possible to observe the change in diameter of a single small axon and the presence of a mitochondrion in the most dilated region.

Serial transverse sections were cut at 0·85 μ and 36 successive sections were photographed. In these it was possible to follow individual axons and make approximate measurements of the diameter. Table 1 gives some typical results. The fibres are not always round in cross-section; the figure for 'diameter' is the mean of the two diameters. In the segment of the axon where a mitochondrion is present the diameter may be approximately doubled.

TABLE 1

Approximate diameter in microns of five sensory axons in the mesothoracic nerve as seen in successive sections cut at 0·85 μ

Figures in heavy type signify that a mitochondrion is visible within the axon

Axon	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
A	0·45	0·45	0·55	**0·9**	**0·95**	0·75	0·45	0·4	0·45
B	—	—	—	**1·1**	**1·1**	1·0	0·7	0·55	0·4
C	0·45	0·45	0·75	**0·75**	0·8	0·7	0·45	0·45	0·45
D	0·55	0·65	0·75	**1·0**	**1·1**	**0·9**	0·75	0·65	0·55
E	0·55	0·65	0·8	**0·95**	0·75	0·55	0·55	0·55	—

Darkly staining elongated particles are present between the fine axons. As judged with the light microscope some of these are almost certainly mitochondria, and that is confirmed by preliminary observations on electron microscope sections; but there is certainly other lipid-rich material, notably the multiple plasma membranes derived from the Schwann cells (see Discussion).

The nerve is enclosed in a connective tissue sheath, the 'neural lamella' (Scharrer, 1939). This sheath is known to be made up of concentric layers of material resembling the connective tissue of vertebrates (Richards, 1944). Each lamina consists of rows of glycoprotein fibrillae oriented at right angles in successive layers (Baccetti, 1955, 1956). It gives a positive reaction with the periodic acid / Schiff reagent (Wigglesworth, 1955).

Transverse sections through this sheath in the *Rhodnius* nerve, examined with the electron microscope (Smith and Wigglesworth, 1959) showed a series of rather dense fibrillar layers with looser tissue between, in which the fibres were differently oriented. Tangential sections through such a lamina show a dense feltwork of banded fibrils. These had a minimum diameter of about 150 Å and banding with a main periodicity of about 500 to 600 Å, as in the collagen of other groups of animals, and with traces of secondary periodicity between the main bands (fig. 6, L). These observations support the belief that the neural lamella of insects contains collagen (compare Hess, 1958).

Beneath the 'neural lamella' is the 'perineurium', a sheet of nucleate cytoplasm, 0·5 to 1 μ thick, exceedingly rich in filamentous mitochondri which are most readily seen in tangential sections (fig. 6, D, E). From the regio of the nuclei (and sometimes elsewhere) darkly staining cytoplasm ofte extends inwards between the nerve axons (fig. 6, C). This material is indistinguishable from the interaxonal material furnished by the glial cells o Schwann cells within the nerve; indeed, Hess (1958) regards both cell type as the equivalent of the Schwann cells of other animals.

Small tracheae and tracheoles pass obliquely through the substance of th 'neural lamella' and run along the nerve within the cytoplasm of the 'perineurium' (fig. 6, C, E). They do not penetrate more deeply into the substanc of the nerve.

DISCUSSION

Fusion of axons. Pringle (1938) produced evidence that the axons from th groups of campaniform organs on the legs of *Periplaneta* fuse to form a compound axon. The rough counts of sensilla on the legs of *Rhodnius* and of smal axons in the corresponding nerves suggest that this does not apply to the grea majority of the small axons coming from the tactile hairs in this material. On the other hand, there is certainly extensive fusion of axons from the sense cell of the antenna; at least 15 sensory axons, and probably more, unite to form a single axon.

Perhaps this reflects a difference in function. In the case of tactile organ on the surface of the body, accurate localization will be desirable; wherea each antenna may function largely as a whole.

Mitochondria in axons. The axons are very rich in mitochondria which ar exceedingly varied in size and shape. The presence of abundant mitochondri apparently streaming out from the body of the sense cell during the regeneration of an axon (fig. 2, C, D), suggests that many of the mitochondria may be produced within the cytoplasm of the nerve-cell body. But the 10- to 20-μ chains of recently, or partially, divided mitochondria seen in some moto axons (fig. 6, F, G) shows that the mitochondria clearly multiply in the axoplasm. (It may be recalled that the 'neurofibrillae' of Apathy in the leech have been shown by Meyer (1957) to be mitochondria; these may be as much a 9 μ in length, 30 times as long as they are thick.)

There has been much argument in the past as to whether the beade appearance seen in stained preparations of axons are pre-existing structure or artifacts (see Meyer, 1955). Many such swellings are doubtless artifacts but the observations recorded here (figs. 1, C; 2, A, B) show that the fusiform enlargements present in the finer axons represent the sites of mitochondria These mitochondria do not come close to the wall of the axon but are separate from it by the neurofibrillae of the axoplasm pressed aside by the particle.

Interaxonal material. The substance between the axons is contributed by the glial or Schwann cells ('Bindegewebszellen' of Schlote (1957), 'lemnoblasts' of Edwards and others (1958)). Electron microscope studies have shown that this material consists of invaginated plasma membranes of the Schwann cells, like the myelin sheath in the nerves of vertebrates. Double membranes of one sort or another are among the commonest components which cause cell structures to stain deeply with osmium / ethyl gallate (Wigglesworth, 1957). Their presence in the interaxonal material doubtless explains much of the coloration observed; but mitochondria are also present and there may be diffusely staining material comparable with the abundant 'grana' described by Schlote (1957) in *Helix*. Hess (1958) has pointed out that in many places the smaller axons in *Periplaneta* are separated by Schwann cell cytoplasm without multiple plasma membranes. That would agree with the light microscope observations recorded here (and with preliminary observations with the electron microscope).

Neural lamella and perineurium. The connective tissue sheath of the insect nerve (the neural lamella) is generally regarded as the product of the underlying cytoplasm (the perineurium) (Scharrer, 1939). Indeed, it is regarded by Edwards (1958) as the basement membrane of the 'lemnoblasts' (= 'glial cells' or Schwann cells). He considers that all cells are capable of laying down such a basement membrane and points out that the basement membrane of invading tracheae fuses with this substance, which is continuous also with the sarcolemma and the basement membrane of the epidermis. But, as pointed out earlier (Wigglesworth, 1956), it may well be that the haemocytes, which discharge PAS-positive granules to form the indefinite connective tissue of insects, may contribute to the substance of the neural lamella. Hess (1958) hints at such a possibility; and Johansson (1957) observed haemocytes intimately associated with this sheath in *Oncopeltus* (Hemiptera), as did Schlote (1957) in *Helix*.

There seems little reason to doubt the conclusion of Edwards (1958) and Hess (1958) that the cells (lemnoblasts, Schwann cells) within the nerve, which provide the inter-axonal sheaths, have the same provenance as the cells at the surface of the larger nerves which do the same thing and at the same time apparently form the neural lamella. But the fact remains that as the nerves approach the ganglia there is a progressive specialization of the cells at the surface to form a nucleated perineurium (fig. 6, D, E), and this specialization is still more evident in the central ganglia themselves.

Enzyme distribution and permeability relations. Exposure of the living intact ganglia and nerves to a solution of 5-bromoindoxyl acetate reveals the presence of a non-specific esterase in the perineurium of *Rhodnius*; but the substrate fails to reach the abundant cholinesterase located in the interaxonal material (Wigglesworth, 1958).

Exposure of the living nerves to neotetrazolium in the presence of succinic acid reveals the presence of succinoxidase in the perineurium and particularly n the thick sleeve of nucleated cytoplasm around the nerve-endings in the ventral muscles (Wigglesworth, 1956).

These observations suggest that the neural lamella is readily permeable and that the effective barrier to the entry of ions, &c. (Hoyle, 1952; Twarog and Roeder, 1956) resides in the cytoplasmic layer (perineurium) below (cf. Edwards and others, 1958). The same conclusion may apply to the nerve-endings in muscle.

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The Histology of the Nervous System of an Insect, *Rhodnius prolixus* (Hemiptera)

II. The Central Ganglia

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With four plates (figs. 1, 2, 6, 7)

SUMMARY

The central nervous system of *Rhodnius* has been studied in sections stained with osmium / ethyl gallate. The single type of glial cell responsible for perineurium and axon sheaths in the peripheral nerves becomes differentiated in the thoracic ganglia into four types.

Type i cells form the layer of perineurium cells filled with filamentous mitochondria. These cells which are rich in succinoxidase control the passage of all substances to and from the ganglion.

Type ii cells produce the thick myelin-type sheaths for the lateral group of motor axons (see below).

Type iii cells, the giant glial cells in the ganglionic layer, have a very extensive cytoplasm which sends deep tongue-like invaginations into the large ganglion cells. This cytoplasm must control the transmission of nutrients and excretory substances to and from the neurones. It contains some non-specific esterase.

Type iv cells form an investment of the neuropile. They also have extensive cytoplasm which surrounds the axons and dendrites and provides intensely staining inter-neuronal material in the synaptic regions. This material, the 'darkly staining neuropile', is the chief site of acetylcholine esterase.

The nerve-tracts are well displayed by the osmium / ethyl gallate method. The axons appear as unstained cords containing mitochondria, against the dark neuropile. The large motor axons of the legs separate into two groups. The medial group run directly into the neuropile and have thin sheaths. The lateral group remain outside the neuropile; they become narrow and enclosed in a thick myelin-type sheath; but as they turn into the neuropile the sheath becomes thin and the axons enlarge again.

THIS paper describes the results of applying the osmium / ethyl gallate method of fixation and staining to the central nervous system in *Rhodnius prolixus*. As already reported (Wigglesworth, 1957, 1959) this method reveals predominantly those structures which are most rich in unsaturated lipids, notably phospholipids. It has therefore proved particularly informative when applied to the central nervous system with its high content of these substances.

There is an extensive literature on the anatomy and histology of the insect nervous system. So far as the Hemiptera are concerned, the anatomy of the brain, with the location and connexions of the various neurones as demonstrated by the Golgi method, has been fully described by Pflugfelder (1937); and the anatomy and histology of the central nervous system of *Oncopeltus* (Lygaeidae), with special reference to the neurosecretory cells, have been

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described by Johansson (1957). But comparatively little information exists about the intimate histological structure of the ganglia.

The object of the present work is to describe the various histological elements which compose the central ganglia, without paying much attention to the anatomy of the nerve-tracts. It may be observed in passing, however that the osmium / ethyl gallate method, in which the axons appear pale against a deeply staining interaxonal substance (figs. 1, C; 7, A), may well prove useful for mapping out the course of nerve-tracts. This deeply staining substance is referred to as the 'darkly staining neuropile', although the nerve-fibres themselves, which constitute the 'neuropile' in the strict sense, are for the most part almost unstained.

Rhodnius larvae in the fifth or last larval stage were used. The methods were those described in the first paper of this series (Wigglesworth, 1959).

The general structure of the ganglia

The anatomy of the central nervous system in *Rhodnius* agrees closely with that described in other Hemiptera by Pflugfelder (1937) and Johansson (1957). Behind the brain and suboesophageal ganglion is a separate prothoracic ganglion (fig. 1, C); the mesothoracic, metathoracic, and all the abdominal ganglia have fused to form a single shield-shaped mass concentrated in the thorax.

Fig. 1, A, B represents a horizontal section through the fused ganglia of the thorax after fixation and staining with osmium / ethyl gallate, and fig. 3, A shows a semischematic drawing of the same structure.

The *ganglion cells* (motor and association neurones), together with the *neurosecretory cells*, are peripheral. The central core of the ganglion is occupied by the darkly staining *neuropile*. This is traversed by the unstained *motor* and *sensory axons*, often collected into nerve-tracts, and their collaterals. Investing the darkly staining neuropile is a covering of *small glial nuclei* lying in a thin pale layer of very fine nerve-fibres. *Giant glial nuclei*, rather few in number, lie among the ganglion cells and provide the copious *glial cytoplasm* by which

FIG. 1 (plate). A, fused meso- and metathoracic and abdominal ganglia in horizontal section Neuropile dark; ganglion cell layer relatively pale. *e* and *f* show positions of sections E and F below.

B, detail of the same showing entry of mesothoracic leg-nerve. *ma*, medial motor axons entering neuropile; *la*, thick-walled lateral motor axons. Glial cell nuclei (type iv) can be seen in the pale zone around the dark neuropile.

C, horizontal section of prothoracic ganglion, showing large motor ganglion cells (above) with axons entering the neuropile.

D, tangential section (0·85 μ) of perineurium showing nuclei and granular and filamentous mitochondria; also a few fat droplets appearing black.

E, transverse section (0·85 μ) through mesothorax just after entry of nerve (see *e* in A). Above and to right, lateral thick-walled motor axons with glial nuclei (type ii) between them (marked by arrows). In the centre, medial thin-walled motor axons. Below, fine sensory axons.

F, the same, cut further forward (see *f* in A). To the right, lateral motor axons with thick walls and glial cell nuclei (arrows), in layer of fine fibres between dark neuropile and perineurium. To the left, medial motor axons with thin walls in the dark neuropile.

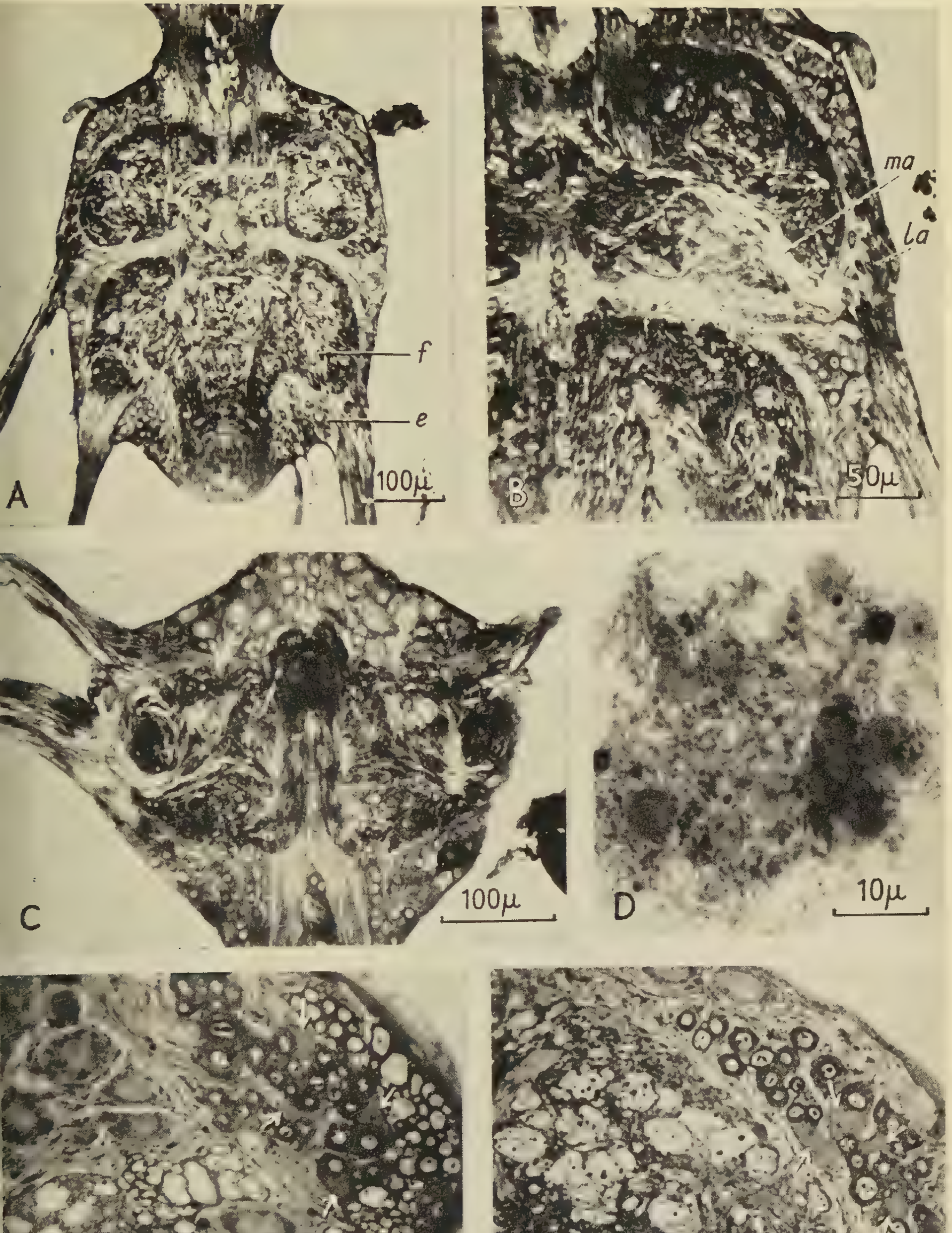

FIG. 1

V. B. WIGGLESWORTH

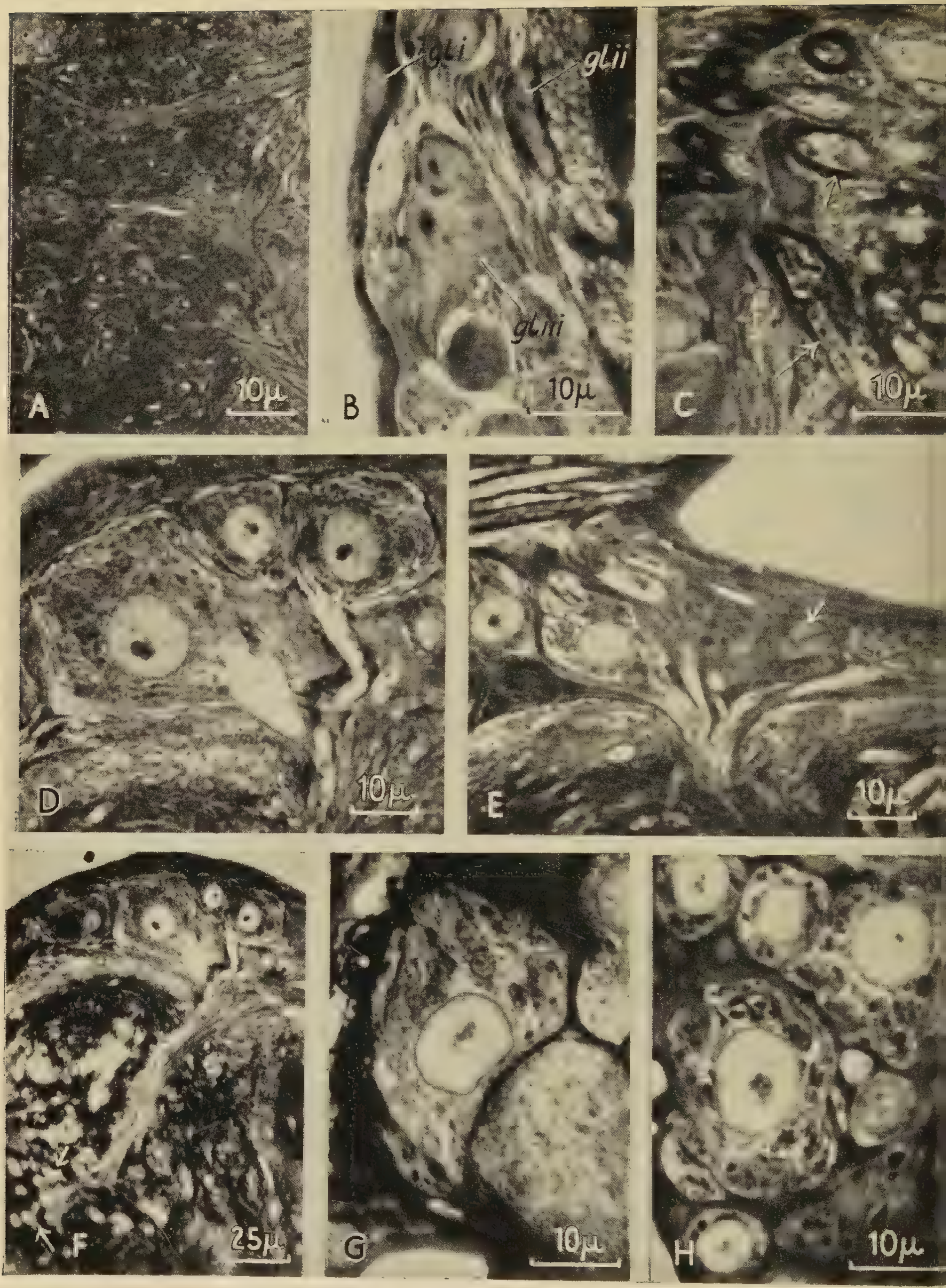

FIG. 2

V. B. WIGGLESWORTH

they are separated from one another and which forms an outer investment of the neuropile. *Sheathforming glial cells* give rise to the thick 'myelin sheath' which invests certain of the motor axons as they run from the neuropile to the nerves. *Tracheal cells* and the tracheae and tracheoles which they form are most plentiful in the ganglion sheath and in the zone investing the neuropile. Finally, the entire ganglion is enclosed in the *perilemma*, consisting of the fibrous *neural lamella* and the *cellular perineurium.*

These elements will be considered in turn, but their close mutual relations make it impossible to describe one entirely independently of another, and this will entail a certain amount of repetition.

The ganglion sheath (perilemma)

The *neural lamella*, the fibrous sheath containing collagen fibrils (Smith and Wigglesworth, 1959), has the same laminated structure as in the nerves; but it is more robust, often as much as 0·75 μ in thickness, and the laminae may be visible even with the light microscope.

The *perineurium* also is better developed. It now forms a cytoplasmic layer some 1·5 to 3·5 μ in thickness, filled with oval and filamentous mitochondria and containing regularly spaced nuclei (fig. 1, D). The tracheae penetrate the ganglion sheath and tracheoles are plentiful in the cytoplasmic layer.

In the larger nerves the cytoplasm of the perineurium may extend deeply inwards and contribute to the interaxonal sheaths (Wigglesworth, 1959). In the central ganglia similar inward extensions of the perineurium are infrequent; for the most part there is a smooth boundary between the inner margin of the perineurium and the glial cytoplasm below (figs. 2, B; 5).

Nerve axons

The *sensory axons*, which occupy the ventral half of the large nerves (Wigglesworth, 1959) enter the neuropile and are there lost and cannot be traced further (fig. 2, A). Sometimes, on entering the ganglion, they come into contact with one of the large glial cells. They may then lie close to the nuclear membrane, and the dark substance of the glial cytoplasm, containing

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FIG. 2 (plate). A, horizontal section (0·85 μ) through posterior extremity of neuropile of abdominal ganglia showing sensory axons entering from the right.

B, *gl* i, nucleus of perineurium cell; *gl* iii, nucleus of giant glial cell with invaginations of nuclear membrane and sensory axons in the cytoplasm; *gl* iv, nucleus of glial cell sending dark processes between axons into the dark neuropile, to the right.

C, above and to left, thick-walled motor axons in oblique transverse section. In centre and below, axons entering the dark neuropile (marked by arrows): sheath becoming thin; massed mitochondria. To the right below, dark neuropile.

D, transverse section (2 μ) of metathoracic ganglion showing large motor cells with axons entering neuropile.

E, horizontal section of metathoracic ganglion showing motor cells with axons entering neuropile. Arrow marks giant glial nucleus with invaginations of nuclear membrane.

F, as D above; motor axons run inwards and dorsally to join the large motor axons (arrows) seen in transverse section.

G, ganglion cell with invaginated plasma membranes reaching almost to nucleus.

H, ganglion cells with invaginations and other inclusions.

mitochondria, forms dark deposits between them as they run towards the neuropile (figs. 2, B; 6, C).

The *motor axons* occupy the dorsal half of the large nerves. As these approach the ganglion the large motor axons fall into two groups, lateral and medial (figs. 1, B, E, F; 3, A).

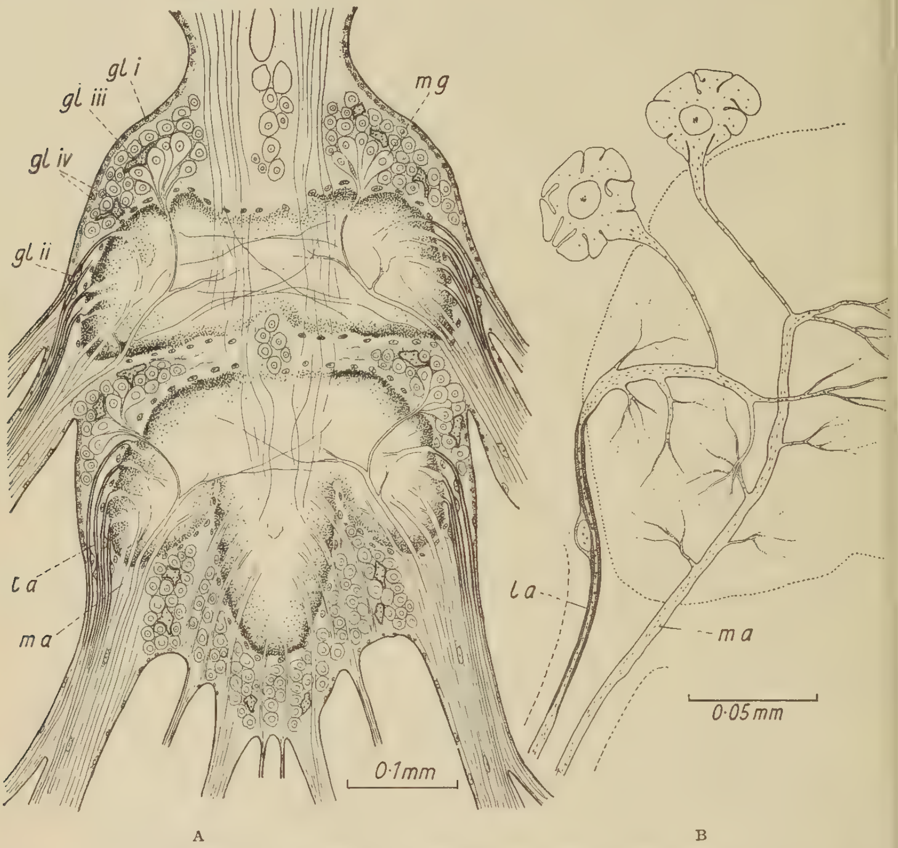

FIG. 3. A, semischematic scale drawing of fused mesothoracic, metathoracic, and abdominal ganglia in *Rhodnius*. *gl* i, perineurium; *gl* ii, glial cells forming sheaths of lateral motor axons; *gl* iii, nucleus of giant glial cell; *gl* iv, nuclei of glial cells surrounding the dark neuropile. *la*, lateral motor axons; *ma*, medial motor axons. *mg*, motor ganglion cells. B, semischematic scale drawing of a typical lateral motor axon (*la*) and medial motor axon (*ma*). The dotted lines mark the boundaries of the nerve and of the dark neuropile.

(*a*) The motor axons of the *lateral group* contract down from a diameter of 4 to 5 μ to a diameter of 1 to 1·5 μ and each becomes invested by a sheath whose thickness may exceed the diameter of the axon (fig. 1, E, F). This sheath stains deeply with osmium / ethyl gallate; it has a concentric structure readily visible with the light microscope, and contains mitochondria. It is the product of the glial cells which are very plentiful among these axons (fig. 5, A, *gl* ii). It may be regarded as a primitive type of 'myelin sheath'.

This lateral group of large motor axons (25 to 30 in number, together with more small axons) pass forward between the neuropile and the ganglion heath. They remain narrow (1 to 1·5 μ) until they turn inwards to enter the neuropile. As they enter they increase abruptly in diameter (to 4 or 5 μ) and he sheath at once becomes thin (less than 0·1 μ) (fig. 2, c). They give off branches to the neuropile in many directions and the axons themselves can be traced to large motor cells in the ventro-lateral region of the ganglion (fig. 3, B).

All these axons contain, as usual, conspicuous mitochondria. In the wide egment of the axon just before this leaves the neuropile and becomes con-tricted, the axoplasm is always filled with a great quantity of globular mitochondria (fig. 2, c). They give the impression that this represents a point of congestion as the mitochondria move down the axons from the cell-body.

In the nerve axons of *Helix*, Schlote (1937) described longitudinal folds of he sheath extending far into the axoplasm. Such folds do not seem to have been described in insects, but they do occur in some of the thick-walled motor axons in *Rhodnius* (fig. 5, B).

(*b*) The motor axons of the *medial group* retain their diameter of 4 or 5 μ unchanged. Their sheath is quite thin (0·1 to 0·2 μ) and they enter the neuropile immediately after reaching the ganglion. They give off small branches at ntervals, which become lost in the darkly staining neuropile, some crossing o the opposite side. They follow a course forwards and towards the mid-line, and narrow abruptly to about 1·5 μ where they join the bundle of axons from he large motor ganglion cells (fig. 3, B).

The foregoing description applies to the group of about 15 to 20 large motor axons. There are many other smaller axons (perhaps 100 to 120 in one of the large leg-nerves) which doubtless follow a similar course to reach smaller motor ganglion cells.

Ganglion cells

Fig. 2, D shows a group of large ganglion cells as seen in a transverse section through the anterior part of the metathorax. This group is lying ventro-lateral; their axons, which are unstained but contain mitochondria, converge upon a point of entry into the neuropile where they form a well-defined tract that joins the large motor axons described above (fig. 2, F). Fig. 2, E shows a similar group seen in a horizontal section, and fig. 1, C shows he large motor cells with their axons entering the neuropile at the anterior part of the prothoracic ganglion.

The axons from these large ganglion cells are at first about 2 to 3 μ in diameter; they narrow down to about 1·5 μ on entering the neuropile, and enlarge again to 4 or 5 μ as they bend in the posterior direction to form the motor-fibres already described (fig. 2, F; 3, B).

When followed into the body of the ganglion cell the axoplasm is seen to fan out into unstained filaments which form an investment of the nucleus (fig. 2, D).

These filaments agree in their distribution with the neurofibrillae as scribed by Beams and King (1932) in the grasshopper *Melanoplus*, by ma authors in silver preparations of vertebrate ganglion cells, and by Eichn (1956) in fresh anterior horn cells of the mouse examined by phase co trast.

The nucleus of the ganglion cell shows a homogeneous or faintly mottl nucleoplasm bounded by a sharply defined nuclear membrane. The nucleol consists of a group of minute black spheres (0·2 to 0·7 μ) in a browni matrix. (Nucleic acids are not stained by osmium / ethyl gallate (Wiggle worth, 1957).)

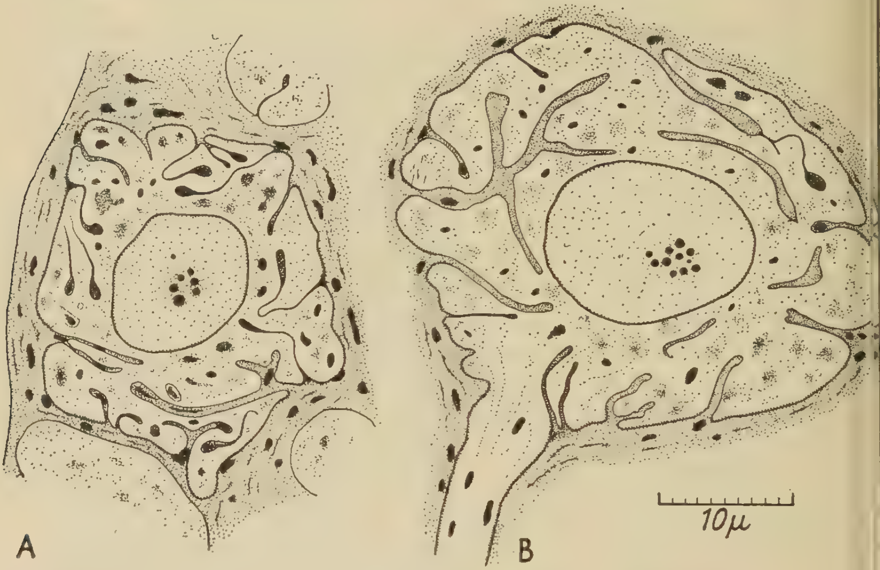

FIG. 4. Motor ganglion cells, surrounded by glial cytoplasm containing mitochondria, sho ing multiple invaginations of the plasma membrane. A, drawn from a single 2μ sectio B, combined drawing from two adjacent 2μ sections.

The larger ganglion cells contain a confusing array of inclusions. These a shown in drawings in fig. 4 and in photographs in fig. 2, D, E, G, H and fi 6, B, D. The most characteristic inclusion takes the form of linear folds finger-like invaginations of the surface membrane which penetrate far into th cell. In some of these invaginations the two surfaces of the plasma membran are in close contact and appear in section as a thin black line which enlarges its inner limit to form a rounded swelling. Not uncommonly these folds a branched or subdivided. In other invaginations the plasma membrane may b separated by 1 μ or more and the intervening space is filled with the gre brown glial cytoplasm to be described later.

When cut in some positions the nature of these invaginations is not im mediately apparent, for they may form crescents (fig. 4, B) or isolated incl sions of varied form with no visible connexion to the cell surface.

The ganglion cells also contain material staining a mottled grey, much of vhich, as seen in electron micrographs, consists of double membranes of endoplasmic reticulum' and, perhaps, the 'Golgi element'. Scattered through he cytoplasm are well-defined oval mitochondria.

The smaller ganglion cells (below about 10 μ diameter) do not show these nvaginations of the cell surface. They contain only the mottled grey material nd the mitochondria which, in the absence of the other inclusions, show up nore distinctly (figs. 2, H; 6, D).

In the supraoesophageal ganglion the cells are nearly all small association eurones with relatively little cytoplasm containing mitochondria. Their fine xons, not more than 0·5 μ in diameter, can be seen streaming into the europile (fig. 6, A).

The *neurosecretory cells* will be considered in the third paper of this series. It nay be noted here, however, that the floccular content of these cells stains brownish colour with osmium / ethyl gallate and this material can be followed little way into the axons (fig. 6, F).

lial cells

In the larger nerves (Edwards and others, 1958; Wigglesworth, 1959) there eems to be no clear distinction between the glial cells (Schwann cells) inside he nerves, which provide the darkly staining axon sheaths, and the peripheral ells, which likewise provide interaxonal material extending into the substance of the nerves, but which also appear responsible for secreting the ibrous neural lamella. Within the ganglia the glial cells become specialized nd differentiated into distinct types (figs. 3, A; 5).

Type i. The cells of the perineurium form a regular investment of the ntire ganglion. They may have the same ontogenetic origin as the other glial ells (that will be considered in the Discussion (p. 309)), but for purposes of escription they have been included under the perilemma (p. 301).

Type ii. As the large nerves enter the ganglia the glial nuclei increase in umber among the lateral motor axons. They give rise to the darkly staining heaths for these axons, which are shown in transverse section in fig. 1, E, F. These axons can be described as 'myelinated'; they may be 1 to 1·5 μ in iameter and the sheath 2 μ thick. Even with the light microscope the sheath s seen to have a concentric structure and contains more darkly staining nitochondria within it (fig. 5, B).

The glial cells responsible for these sheaths occur throughout the course of he lateral motor axons as they run forward between the perineurium and the arkly staining neuropile. As the axons turn into the neuropile and increase in iameter the sheaths become thin (figs. 2, C; 3, B).

Type iii. The third type, the giant glial cell, is peculiar to the ganglia. These ells are few in number but of prodigious size. The nuclei may exceed 60 μ in ength and 15 μ in breadth. The nucleoplasm is coloured a uniform pinkish rown and contains a number of scattered, deeply staining nucleoli. The uclear membrane is invaginated to form deep folds within the nuclear sap (figs. 2, B, E; 6, B, D) and often the nucleus is filled with delicate membrane produced in this way (figs. 5, A; 6, C).

The cytoplasm of the giant glial cells has the same pinkish-brown colour as the nucleus. It spreads in all directions, between the ganglion cells and between the ganglion cell layer and the neuropile (figs. 5, A; 6, B). It contains mitochondria staining a deep blue, and fine darkly staining membranes which appear

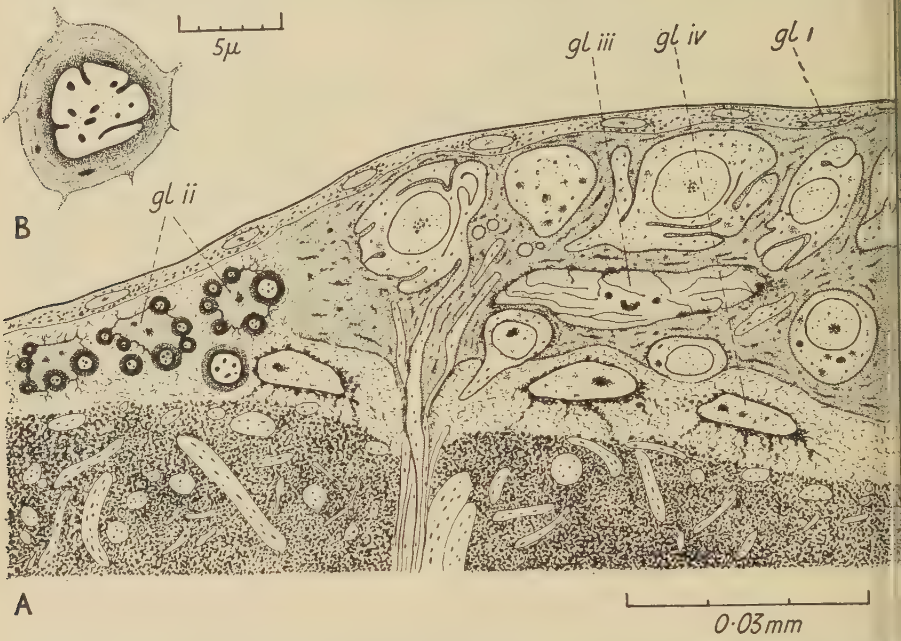

FIG. 5. A, section through margin of prothoracic ganglion to show the different types of glial cell. *gl* i, perineurium with abundant mitochondria in cytoplasm, and neural lamella externally; *gl* ii, glial cell nuclei producing the thick 'myelin' sheath for the lateral motor axons; *gl* iii, giant glial nucleus with invaginated membranes, and cytoplasm containing mitochondria extending everywhere between the ganglion cells and their axons; *gl* iv, glial nuclei in the zone of fine nerve-fibres, sending darkly staining cytoplasm with mitochondria inwards to form the the dark substance of the neuropile. B, section through one of the lateral motor axons showing thick laminated sheath with mitochondria, invaginations of the sheath into the axon, and mitochondria in the axoplasm.

in sections as almost invisibly fine lines oriented in the plane of spreading. Occasional minute blue-black droplets of fat are present. The ganglion cells are often separated by layers of this cytoplasm 3 or 4 μ thick. It is usually darker in tone than the ground substance of the ganglion cells, so that these appear pale against a darker background (fig. 2, G, H). The glial cytoplasm extends in a tongue-like form into the deep folds in the ganglion cells (figs. 4; 5, A; 6, D) and these invaginations may contain mitochondria and other darkly staining material.

When a bundle of fine sensory axons passes by one of the giant glial nuclei, the glial cytoplasm extends between them and accompanies them into the

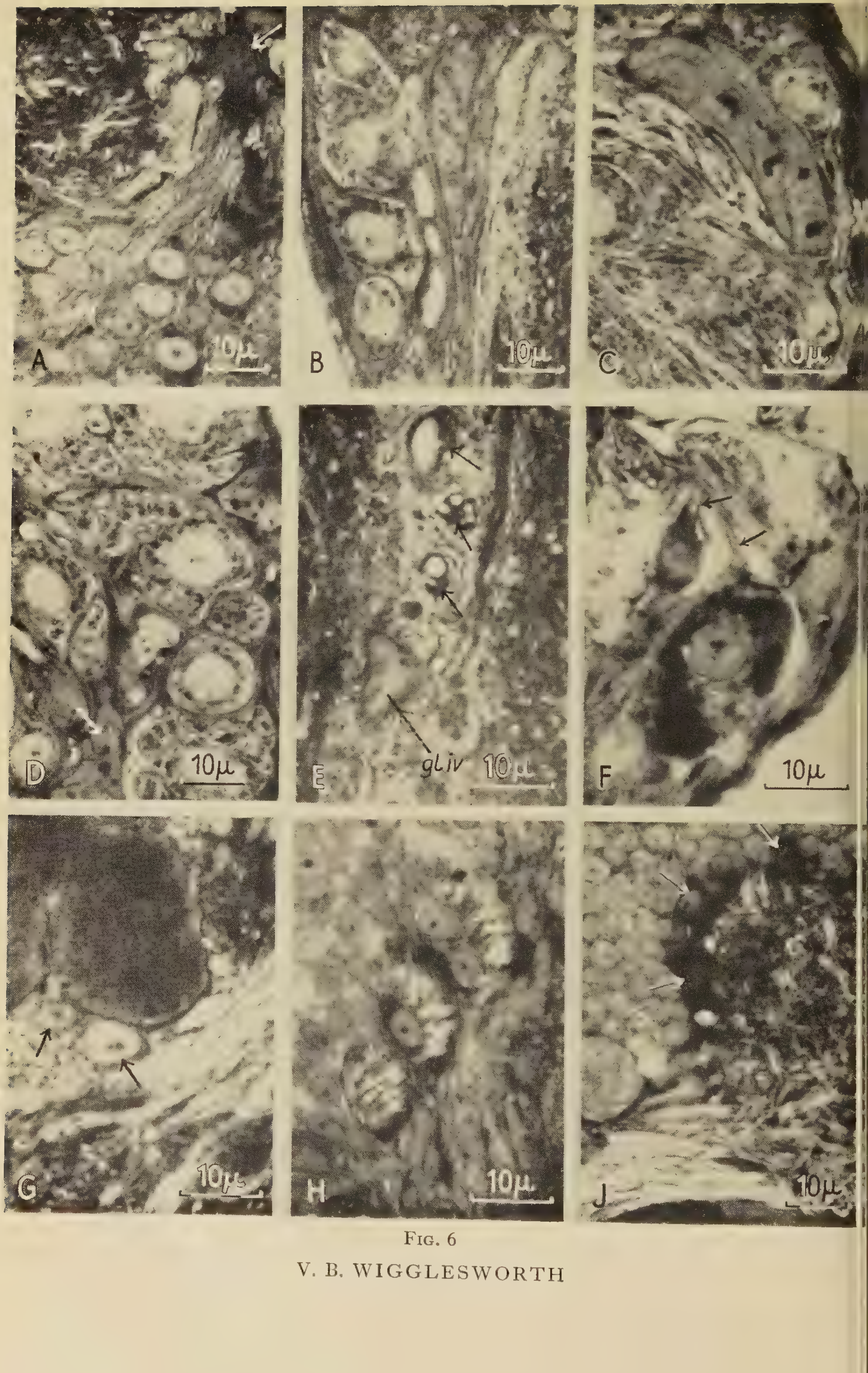

FIG. 6

V. B. WIGGLESWORTH

neuropile (figs. 2, B; 6, C). In the same way, the glial cytoplasm forms a covering for the large motor axons as they run from the ganglion cells into the neuropile (fig. 2, D, E).

Type iv. The fourth type of glial cell has a smaller nuclear size (5 to 15 μ). These cells form a more or less continuous investment of the darkly staining neuropile (figs. 3, A; 5, A), and in the central region, where longitudinal tracts of fine axons are passing through the ganglion, they may be found also in the deeper parts of the neuropile (compare Johansson, 1957).

At first sight these cells appear to have very little cytoplasm. There is just a narrow zone of dark material containing mitochondria in immediate contact with the nucleus. But in favourable sections it can be seen that this cytoplasm is continuous with the darkly staining material which lies between the fine nerve-fibres and accompanies them as they enter the neuropile (figs. 2, B, 5, E). Indeed, there is little doubt that these glial nuclei provide the darkly staining interneuronal material which is such a conspicuous feature of the neuropile. This will be discussed more fully in the section dealing with the neuropile.

In the brain there is some further specialization of glial cells. The perineurium (type i) is identical with that in the thoracic ganglia; and there are a few giant nuclei (type iii) present. In some regions of the neuropile there are cells closely resembling those described in the thorax as type iv, and these give rise to the dark substance in the neuropile (fig. 6, A). The more specialized regions of neuropile (the optic ganglia, mushroom bodies, &c.) are surrounded by an investment of glial cells of a somewhat different type. The nuclei are quite small (4 to 7 μ), the cytoplasm a uniform dark grey. This dark cytoplasm penetrates between the nerve-fibres to form a darkly staining reticulum throughout the neuropile (fig. 6, G, H, J).

FIG. 6 (plate). A, section of brain (2 μ) showing fine axons from association neurones entering the neuropile. An arrow marks a glial cell with cytoplasm continuous with the dark neuropile (top left).

B, in the centre, giant glial cell nucleus with invaginated membranes. To left, glial cytoplasm with mitochondria, enclosing motor ganglion cells and axons; perineurium with mitochondria; perilemma. To right, glial cytoplasm; pale layer of fine nerve-fibres; dark neuropile.

C, giant glial cell nucleus with nucleoli and invaginated nuclear membranes; cytoplasm of glial cell filled with sensory fibres.

D, bottom left (marked with arrow), giant glial cell nucleus with invaginations in nuclear membrane. Glial cytoplasm with mitochondria extends between the ganglion cells and is invaginated into them.

E, horizontal section between dark neuropile of meso- and metathoracic ganglia. Arrows mark nuclei of tracheal cells with tracheae and tracheoles. *gl* iv, nucleus of glial cell.

F, neurosecretory cells in thoracic ganglia with axons marked by arrows.

G, in the centre, two glial cells (arrows) sending darkly staining cytoplasm into neuropile of corpora pedunculata.

H, glial cells sending cytoplasmic sheets with mitochondria among the bundles of axons (unstained) to the optic ganglion.

J, optic nerve (below) enters the optic ganglion (to right). Ring of glial cells (marked with arrows) send dark cytoplasmic processes into the neuropile.

The neuropile

The central core of the ganglion, which is devoid of ganglion cells, is termed the neuropile; but this varies greatly in its histological character in different parts. It contains large and small axons, often collected into bundles or nerve-tracts and often branching. The axoplasm of these fibres is almost unstained but contains great numbers of mitochondria, sometimes rounded, usually elongated in the long axis of the fibre (fig. 7, A, B). Even small fibres, 0·5 μ or less in diameter, contain such mitochondria, but they enlarge up to a diameter of about 1 μ at the point where the mitochondrion lies (Wigglesworth, 1959).

All the nerve-fibres are surrounded by rather thin but darkly staining sheaths, and occasional mitochondria are seen in the substance of the sheath. Where the nerve-fibres are small there is relatively more sheath material and the neuropile appears darker; but those regions consisting mainly of fibre-bundles never stain very deeply.

A large part of the neuropile, however, consists of intensely staining material with a deep grey coloration (fig. 7, B). It is this which has been referred to throughout this paper as the 'darkly staining neuropile'. The dark neuropile probably represents the synaptic regions. The nerve-fibres are all small (usually less than 1 μ); they are no longer in bundles but form an intricate tangle. They contain deeply staining mitochondria, and in many of them the axoplasm stains a grey shade. This is doubtless due to the presence of the so-called 'synaptic vesicles' which are evident in electron microscope sections of these regions (fig. 7, F).

But the main cause of the dark staining is the presence of irregular flake-like masses of dark material between the fine nerve-fibres. This material contains still more deeply staining mitochondria and closely resembles the cytoplasm of the glial cells (type iv) which surrounds the glial nuclei.

In many sections there is no obvious connexion between the dark substance and these glial cells. But the glial nuclei are large and have almost no obvious cytoplasm around them. It is highly probable that the cytoplasm is actually very extensive but reaches far away from the nuclei. Indeed, in favourable sections it is not difficult to confirm this impression and to see clearly the dark

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FIG. 7 (plate). A, section of neuropile (0·85 μ), showing branching axons of all sizes with mitochondria in the axoplasm, and dark material with mitochondria between.

B, neuropile (0·85 μ section) with nerve-fibres of all sizes. To the left, the darkly staining neuropile.

C, glial cell (type iv) showing cytoplasm continuous with the darkly staining neuropile. Nerve-fibres in transverse and longitudinal section.

D, the same.

E, electron micrograph of neuropile (by Dr. David S. Smith) showing small nerve-fibres in transverse and longitudinal section.

F, electron micrograph of neuropile (D. S. S.) showing mitochondria and synaptic vesicles (?) in the nerve-fibres.

G, electron micrograph of dark neuropile (D. S. S.) showing cytoplasm of glial cells, with multiple membranes and mitochondria, between the fine nerve-fibres.

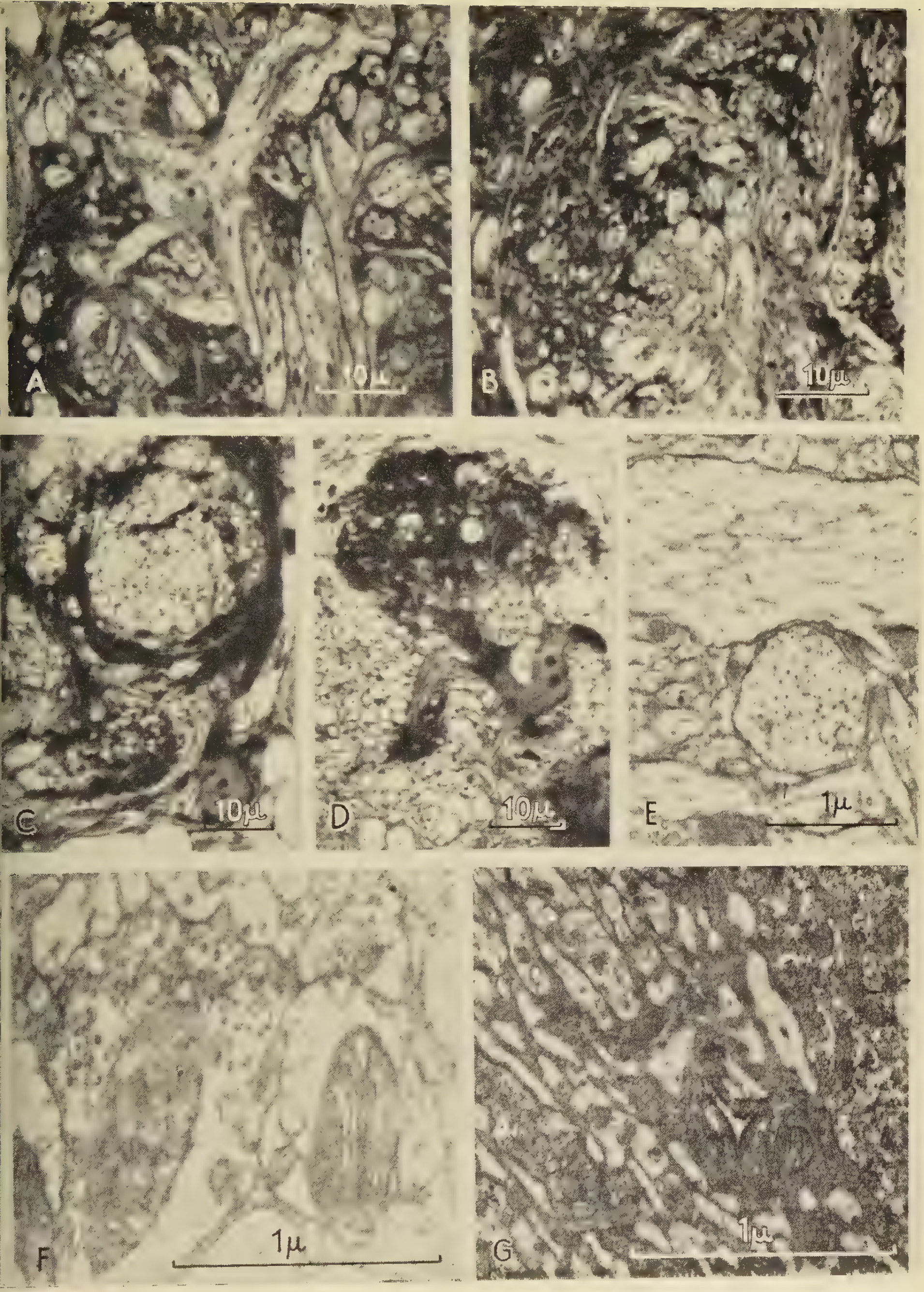

Fig. 7

V. B. WIGGLESWORTH

ubstance of the glial cytoplasm, with its mitochondria, extending between he axons and becoming continuous with the dark material which composes he darkly staining neuropile. Fig. 7, C, D show examples of this.

In the brain, the character of the darkly staining neuropile (in the optic anglia, mushroom bodies, central body, etc.) varies considerably in different arts. But here also it is not difficult to establish the continuity of the dark nterneuronal material with the cytoplasm of the glial cells which are applied o the outer surface of the neuropile (fig. 6, G, H, J).

Tracheae and tracheoles

The general tracheal supply of the thoracic ganglia of *Rhodnius* has already een figured (Wigglesworth, 1954). The tracheae penetrate the sheath, their asement membrane fusing with the neural lamella (compare Edwards and thers, 1958). There is a rich supply of tracheoles within the perineurium, hich emphasizes the metabolic activity of these cells. Within the ganglia he tracheal supply is most rich around the surface of the neuropile. Perhaps his indicates the high oxygen requirements of the type iv glial cells which maintain the interneuronal cytoplasm of the neuropile.

Fig. 6, E is a horizontal section through the zone of pale neuropile between he meso- and metathoracic ganglia. It shows the small tracheae breaking up nto tracheoles, seen in transverse section as pure white spots in immediate ontact with small tracheal cells. The glial cells are quite separate from the racheoles and are clearly not concerned in their formation.

Electron microscope sections of the neuropile

The fine structure of the neuropile will require detailed study with the lectron microscope. A few preliminary sections were kindly prepared for me y Dr. David S. Smith, and three small fields are here reproduced.

Fig. 7 F shows a number of nerve-fibres ranging from 0·1 to 1 μ in diameter. These contain mitochondria, often with longitudinal cristae, which may be as much as 1 μ in length. In addition, some of the nerve-fibres contain the so-alled 'synaptic vesicles' which are doubtless responsible for some of the smiophil coloration of the neuropile.

Fig. 7, G shows what is evidently a part of the most darkly staining neuro-ile. In between the nerve-fibres, most of which are in the 0·1 to 0·25 μ ange, there is a strongly osmiophil substance containing numerous mitochon-ria, double membranes of varied arrangement, and indefinite granular material. It is this substance which is regarded as the cytoplasm of the glial ells (type iv) and is responsible for most of the dark staining with osmium / thyl gallate.

DISCUSSION

Perineurium and neuroglia

In a very useful paper on the ganglia of the cockroach, Scharrer (1939) emonstrated a clear distinction between the perineurium cells and the glial cells. The perineurium cells do not have processes extending between th neurones, and they alone take up trypan blue injected into the living insect The glial cells have multiple ramifying processes, and contain proteinaceou 'gliosomes', glycogen, and fat.

Although 'gliosomes' are absent in *Rhodnius*, as in *Oncopeltus* (Johansson 1957) there is an equally clear-cut distinction between perineurium and glia cells in the ganglia. The perineurium cells, which are probably responsible fo secretion of the collagenous neural lamella (though contribution by the haemocytes remains a possibility (Wigglesworth, 1956)), are characterized by a abundance of filiform mitochondria and a high content of succinoxidase an non-specific esterase (Wigglesworth, 1958). They doubtless function as a active barrier between haemolymph and nervous tissue. All the nutritive an excretory exchanges between the ganglia and the haemolymph take plac through the perineurium, which must therefore have a singularly importan role in metabolism.

It is obvious that this role will be greater in the ganglia, with their larg size and many cells, than in the peripheral nerves; and greater in the larg nerves than in the small. That is the probable reason for the progressive development of the perineurium as the nerves enlarge and join the ganglia In the smallest nerves there is no distinction between glial cells and perineurium; the same cell does duty for both (Edwards and others, 1958 Wigglesworth, 1959). In the larger nerves, although a perineurium rich i mitochondria is present, it still contributes to the interaxonal sheaths (Wigglesworth, 1959). In the ganglia, specialization is complete and the perineurium sends almost no extensions between the underlying neurones.

It therefore seems probable that the perineurium cells are derived from glial cells which have become specialized for the function of providing protective, nutritive, and excretory sheath for the larger nerves and the ganglia This specialization involves an increasingly rich tracheal supply and th presence of great numbers of mitochondria. A 'trophic function' was one o several suggestions put forward by Steopoe and Dornesco (1936).

The glial cells of type ii, which give rise to the sheaths of the lateral moto axons, call for little discussion. The chief points of interest about these are th great thickness of these laminated sheaths (they are relatively much thicke than the myelin sheath in many vertebrate nerves) and the fact that such sheaths occur only around the large axons which run outside the darkly staining neuropile, and become quite thin again as soon as the axons enter the dark neuropile. There is no reason to doubt that these sheaths are of the same general nature as the myelin sheath of vertebrates; in the central nervous system of vertebrates the protoplasmic sheets spreading from the glial cells around the axons may contain cytoplasm and mitochondria as well as the multiple plasma membranes (Palay, 1958; de Robertis, Gerschenfeld, and Wald, 1958).

The giant glial cells (type iii) have previously been described in *Apis* (Weyer, 1931; Risler, 1954), *Hydrous* (de Lerma, 1949), *Musca* (Grandori and

ers, 1951), *Oncopeltus* (Johansson, 1957) and other insects. In Hymenop-
a (Risler, 1954) and also in Lepidoptera there are highly polyploid
cheal end-cells within the ganglia, but these are quite distinct from the
al cells (Risler, 1954). In *Rhodnius*, as in the cockroach (Scharrer, 1939),
which the tracheal cells are quite small, there can be no confusion between
e two; the tracheal cytoplasm makes a negligible contribution to the
asma between the neurones; whereas in *Bombus*, Meyer (1956) believes
at the peritracheal cytoplasm forms an additional glial component of some
portance. In *Rhodnius*, as in vertebrates (Lumsden, 1958), the 'ground
bstance' between the ganglion cells is the cytoplasm of the giant neuroglial
lls.

These cells are commonly described as having a supporting or trophic
nction. There can be little doubt that their function is nutritive in the widest
nse. Their cytoplasm is the intermediary for the passage of all nutritive and
cretory products to and from the ganglion cells. This cytoplasm is weakly
AS-positive (Wigglesworth, 1956); it contains mitochondria, occasional
inute droplets of fat, and doubtless other inclusions. The boundary mem-
ane is invaginated deeply into the cell-bodies of the large ganglion cells (the
ophospongium' of Holmgren, 1900; compare Beams and King, 1932;
eopoe and Dornesco, 1935), and these invaginations may also contain mito-
ondria and other inclusions, and are the site of a non-specific esterase
Vigglesworth, 1958). It is interesting that in the large glial cells the nuclear
embrane is similarly invaginated.

The question immediately arises as to how stable these various invagina-
ons (figs. 4, 5) may be. Do they represent more or less permanent structures
the cell or are they continually in motion and in the nature of a 'pinacytosis'
volving the engulfment of cytoplasmic contents from the glia? No answers
these questions can be given; but no histological appearances have been
en to suggest that the ends of the invaginations are in fact 'pinched off' into
e cytoplasm of the ganglion cell.

The glial cells (type iv) which lie around the periphery of the neuropile,
hen stained by silver methods, show highly branched fibrils running inwards
ánchez y Sánchez, 1935; Scharrer, 1939). But the osmium / ethyl gallate pre-
rations give rather the appearance of a continuous ensheathing membrane
tween the nerve-fibres, which is thickened to form flake-like masses in the
naptic regions. The provision of this deeply staining interneuronal cyto-
asm, containing double membranes, fine granules, and mitochondria,
ems to be the chief function of the type iv glial cells. This material is the
e of the abundant acetylcholine esterase of the neuropile (Wigglesworth,
58).

The neuropile of insect ganglia contains material staining diffusely with
dan colorants (Wigglesworth, 1942). It was suggested by Richards (1943)
at this is probably bound phospholipid. There is little doubt that it is this
me material, composed largely of plasma membrane sheaths, which is
ponsible for the intense staining with osmium / ethyl gallate.

Ganglion cells and nerve-fibres

Insufficient electron microscope sections of the ganglion cells have been examined to identify with certainty all the inclusions present. Many of these are fragments of the invaginated plasma membranes enclosing mitochondria and other components of the glial cytoplasm. The mitochondria of the ganglion cell itself can generally be recognized. Then there are darkly staining areas with less definite outlines and occasionally with small vacuoles; these are probably the massed systems of double membranes which have been identified with the Golgi element. Finally, there are slightly larger dark objects which may perhaps be comparable with the 'oval bodies' described by Meyer (1956) in the ganglion cells of *Apis*; these are presumably composed mainly of phospholipids and have the most diverse internal structure (compare Revel, Ito, and Fawcett, 1958).

As the axons enter the large ganglion cells the axoplasm is spread out in the form of a meshwork around the nucleus (fig. 2, D). These unstained filaments resemble the strands termed 'neurofibrils' in silver preparations (Beams and King, 1932). In the axons themselves the term 'neurofibrils' is commonly applied to the fine osmiophil filaments, which perhaps represent endoplasmic reticulum, that run a more or less parallel course along the fibre. If true neurofibrils exist in the axons, it is perhaps more likely that they occupy the clear spaces in between the dark filaments (see, for example, the transverse section of a small axon as shown in the electron microscope section, fig. 7, G).

I am indebted to Dr. David S. Smith for the preparation of some thin sections for the electron microscope. It is a pleasure to acknowledge the patient assistance of Mr. F. J. Bloy with the large amount of photomicrography.

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A Simple Method for Cutting Sections in the 0·5 to 1 μ Range, and for Sections of Chitin

By V. B. WIGGLESWORTH

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With one plate (fig. 2)

SUMMARY

Tissues fixed and stained with osmium/ethyl gallate often require sectioning at 0·5 to 1 μ. In the method described they are double-embedded in agar and 'ester wax' (Steedman), cut on a film of water on the coverslip, and mounted in gum containing ethyl gallate. Prepared safety razor blades are recommended. This method is useful also for cutting serial sections of insect cuticle.

THE osmium/ethyl gallate method for histological fixation and staining (Wigglesworth, 1957) gives such intense coloration in most tissues that sections must not exceed 2 to 3 μ; and in tissues such as the liver, where the cells are densely packed with mitochondria, sections must be only 0·5 to 1 μ thick if a confused mass of superimposed mitochondrial granules is to be avoided.

In the development of this staining method the sections were usually cut in polyethylene glycol wax and mounted individually. The purpose of this note is to describe a simple procedure by which serial sections in the 0·5 μ range can be prepared.

The methods and the embedding media for section cutting in the 1 μ range have been fully reviewed by Gettner and Ornstein (1956) and I have made extensive use of their article. The embedding medium employed is the 'ester wax' of Steedman (1947) as supplied by British Drug Houses Ltd. (B.D.H.); equally good results are obtained with the modified 'ester wax' of Chesterman and Leach (1956).

The tissue is first embedded in 5% agar (Haarløv and Weis-Fogh, 1952) and the agar block then embedded in ester wax. The agar improves the elasticity of the block and reduces compression. It is also convenient for orientation and it increases the affinity for water so that the ribbon spreads rapidly and steadily as soon as it is moistened.

The standard Cambridge rocking microtome has been used. In this model one tooth on the milled wheel gives an advance of 0·42 μ; this is as thin as is generally useful for the light microscope. Tolerably complete series have been cut in dry ribbons at 0·85 and 0·42 μ, but it is more convenient to cut directly on to a moist film on the coverslip.

Special care is needed to prepare a standard microtome knife to cut at this thickness without scratches. It is much simpler to use safety razor blades

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prepared and mounted by a slight modification of the method of Sjöstrand (1956), which is described in the appendix to this paper.

Sections prepared by this method have been figured in some recent papers on the insect nervous system (Wigglesworth, 1959 *a*, *b*).

PROCEDURE

Fixation. Small pieces of tissue, 1 to 2 mm in thickness, are fixed in 1% osmium tetroxide in isotonic veronal buffer at pH 7·2 (Palade, 1952) for 4 h. They are then rinsed in distilled water.

Staining. The tissue is transferred to a saturated solution of ethyl gallate (progallin A of Nipa Laboratories) in 0·25% cresol (as a preservative) for 16 to 24 h.

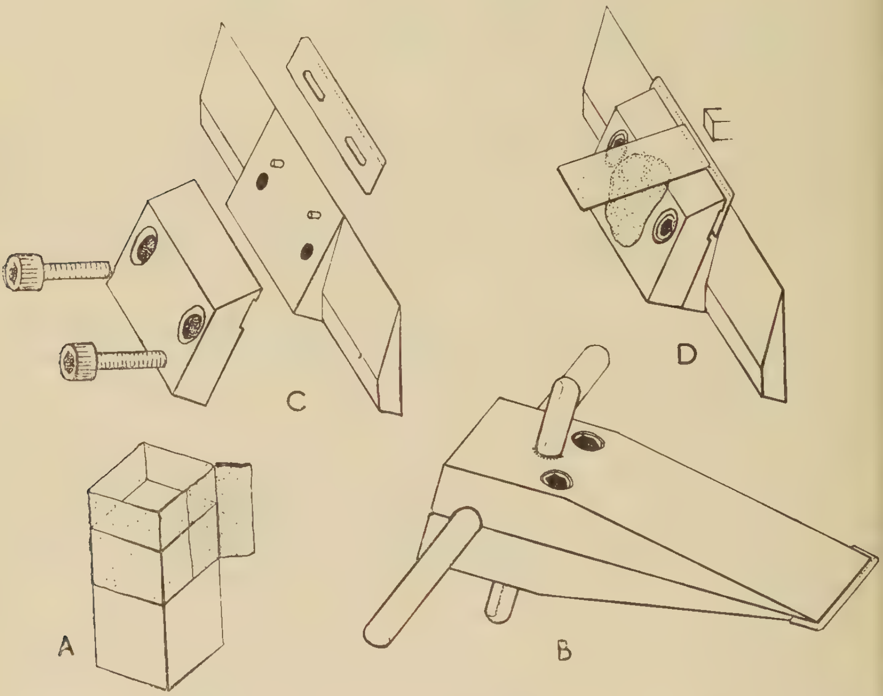

FIG. 1. A, brass block bound with cellophane tape to form embedding trough. B, blade holder for use during sharpening, with pegs to give 40° facet inserted. C, blade-holder for use during cutting. D, the same assembled, with half-coverslip in position to receive the sections.

Embedding in agar. The tissue is placed in 5% agar at 55 to 60° C for 1 h or longer. The agar is then allowed to set at room temperature and it is at this stage that the object is orientated.

This is conveniently done as follows (fig. 1, A). Pieces of brass rod with square cross-section (7 to 9 mm) are cut into lengths of 10 to 15 mm. The metal block so formed is bound with adhesive cellophane tape so that this projects 3 mm above the surface to form the embedding trough. For easy removal it is advisable to cut the free end of the tape and bend it over to form a small tab; and also to apply a square of cellotape to the floor of the trough.

The metal block is heated by standing on the embedding bath and the trough is filled with agar containing the specimen. The specimen is now orientated under the binocular with the face that is to be cut lying against the floor of the trough, and the agar allowed to set.

The cellophane tape is removed and the agar block trimmed until it is rather larger than the block that will finally be cut.

Embedding in ester wax. The agar block is passed through the following series (half-an-hour in each): ethyl alcohol 30%; 50%; 70%; 70% alcohol plus cellosolve, 2:1; the same, 1:2; pure cellosolve (3 changes); cellosolve plus ester wax, equal parts; pure ester wax, at least two changes. It is left overnight at 55 to 60° C in the final ester wax.

The agar block is now placed in the same type of embedding trough as before, with the face that is to be cut resting on the surface. Since the specimen was oriented in the agar no further orientation is necessary. The trough is filled with ester wax and cooled rapidly by standing in a Petri dish in a fast stream of cold water, with the usual precautions to avoid the inclusion of air (Steedman, 1947).

Dry sections. The block is mounted and trimmed so that the agar extends to the surface on all sides. (If a margin of ester wax alone is left, it will restrict flattening on that side.) The sections are cut slowly as with pure ester wax.

As with pure ester wax the sections are apt to become separated and scattered during flattening, when several rows are placed on one slide. It is therefore helpful to prepare the slide as follows. First smear with glycerine-albumen. Then wrap a wisp of cotton wool round the points of a fine forceps, dip the point in yellow soft paraffin, trace out on the slide the margins of the area to be occupied by the sections, and divide this by horizontal lines to form a compartment for each row. Lay the ribbon in its compartment; apply a small drop of 20% alcohol to one end of the compartment and let it flow below the ribbon to the other end. Immediately the drop reaches the ribbon this unfolds smoothly and steadily. Any excess fluid is removed with a pipette and final flattening and drying completed on the warm plate. The sections shown in fig. 2, A–D were cut dry in this way.

Sections on a moist film. When cutting sections at 1 μ or less it is simpler to collect the ribbon directly on to a film of water. A half-coverslip measuring 11 by 22 mm is used. A mound of 'plasticine' is built up on the knife holder to such a level that a coverslip placed on it and resting just below the edge of the blade, will be horizontal (fig. 1, D).

The upper surface of the coverslip is smeared with glycerine-albumen, and the lower surface with yellow soft paraffin.

A section or two sections are cut dry and left adhering to the edge of the blade. (This is essential in order that the water shall be separated from the front of the block. If the front of the block or the upper surface of the section is allowed to get wet the section will be picked up when the block moves up again behind the blade.)

About 8 or 10 drops of distilled water containing 0·1% sodium chrom-glucosate (D. W. Haering & Co., San Antonio, Texas) as an anti-corrosive are placed on the coverslip and swept up to the edge of the knife and below the newly cut sections, with a fine camel's hair brush. The sections will probably spread on the water; and on cutting more sections these will spread into an even ribbon with little compression.

If it is desired to place a second length of ribbon on the coverslip, the first ribbon is cut through with a mounted bristle, displaced to one side, and cutting resumed. The operations are best done under a dissecting microscope.

The sections shown in fig. 2, E–G were cut on a film of water in this way.

Mounting. After draining off excess fluid the slides or coverslips are dried on the warm plate to complete the flattening of the sections. They are then immersed briefly in xylene, taken down to 30% alcohol, and mounted in Farrants's medium (or other aqueous gum medium) containing ethyl gallate in solution. The slide is immediately placed on filter paper with the coverslip downwards and pressed very firmly to displace as much of the mountant as possible.

The staining is completely permanent and is improved by leaving on the warm plate exposed to the sun.

RESULTS

Sections stained with osmium / ethyl gallate. Fig. 2, A–D shows sections of mouse liver cut dry at 2 μ, 1·25 μ, 0·85 μ, and 0·42 μ. In the 2 μ section there is a confusion of super-imposed mitochondria. At 0·42 μ there is only a single layer of mitochondrial granules, many of which are cut through. If the sections are less than 0·42 μ the contrast is reduced and they allow no increased resolution in the light microscope. For visual examination, as opposed to photography, sections at 0·85 μ are very satisfactory for this tissue.

Fig. 2, E–G shows sections of the proximal convoluted tubules in the mouse kidney after cutting on a film of water at 2 μ, 0·85 μ, and 0·42 μ. Here again, the 0·85 μ sections are satisfactory for visual examination, but even at this thickness the densely packed palisade of mitochondria overlap and obscure one another in photographs.

For tissues which are not so rich in mitochondria, such as the intestinal epithelium, sections cut at 2 μ are thin enough for many purposes.

Sections stained by other methods. The same methods of embedding and

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FIG. 2 (plate). A–D, sections of mouse-liver fixed and stained with osmium / ethyl gallate. Blood-corpuscles visible in the capillaries; the intensely stained droplets in the cells (deep blue in the section) are fat droplets, the paler granules (grey-brown in the section) are mitochondria. A, 2 μ; B, 1·25 μ; C, 0·85 μ; D, 0·42 μ.

E–G, sections of proximal convoluted tubule of mouse-kidney showing palisade of mitochondria. E, 2 μ; F, 0·85 μ; G, 0·42 μ.

H, horizontal section of posterior region of head of *Rhodnius* invaginated into the prothorax. J, the same in transverse section.

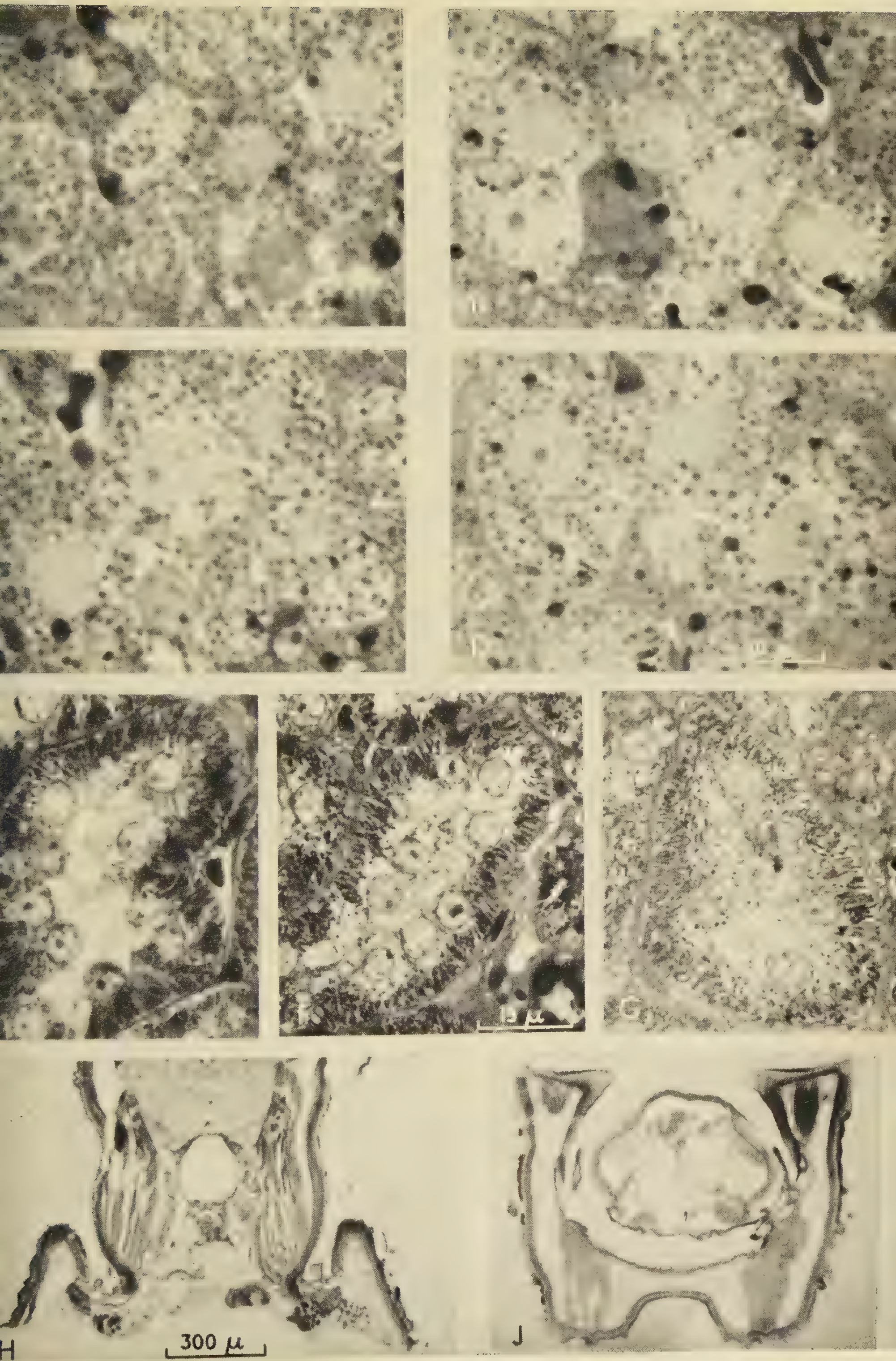

FIG. 2

V. B. WIGGLESWORTH

ection cutting at any thickness can be employed after other fixatives, and ny standard stains can be used. Some of these stains colour the agar film but an be removed by brief treatment with acid alcohol.

Sections of chitin. Double embedding in agar and ester wax has proved ery useful for cutting sections of insect cuticle. Fig. 2, H shows a 6 μ horiontal section of the posterior region of the head in the bug *Rhodnius prolixus*, vhere the neck is invaginated into the front of the thorax. Fig. 2, J shows the ame in transverse section. This is difficult material to cut; it is unusual, by ther methods, to obtain the almost unbroken series with the epidermal cells losely adherent to the cuticle, which are readily obtained by this method.

APPENDIX

Preparation and mounting of the blade

s already pointed out it is possible to cut sections by the methods described with n ordinary microtome knife with a good edge. But it is simpler to use safety razor lades. Blades of the American 'Schick' or the English 'Pal' or 'Personna' type, neasuring 3·75 cm × 8 mm, have been used; and these have been prepared by methods losely based on the procedures described by Sjöstrand (1956) and by Gettner and Ornstein (1956). I have made some small modifications and it may be useful briefly o describe the methods.

Fig. 1, B shows the brass holder for sharpening the blades. This measures 3 × 7 cm. t is of the type described by Sjöstrand but is provided with a series of flat-sided teel pegs of different lengths so as to give facet angles of 25°, 30°, 40°, and 50°. In the figure the 40°-pegs are in use.) By the addition of suitable washers below he pegs it is possible to obtain any facet angle from 25° to 55°. For soft material a acet angle of 35° has generally been used; for harder material an angle of 50°.

To prepare a facet angle of 35°, the 30°-pegs are inserted and the blade is ground down by the method of Gettner and Ornstein on plate glass or on a ground glass tone, by using their lapping fluid consisting of Linde A synthetic sapphire polishing owder (Linde Air Products Co., 30 East 42nd Street, New York 17, N.Y.) 1 volume, n 2 volumes of the following solution: distilled water 10, glycerol 8, acetone 2. After washing the blade and the stone, polishing is continued with the same lapping luid containing Linde B powder. Finally, the blade and holder are washed again, he facet angle is increased to 35° by the insertion of the appropriate washers beneath he pegs, and the blade is very gently polished on another sheet of plate glass with a apping fluid consisting only of distilled water 6, glycerol 12, acetone 2, with no owder. This gives a very small 35° facet at the margin of the 30° edge. The whole rocedure requires only about 10 minutes.

The blade holder for section cutting is illustrated in fig. 1, C, D; the drawings are self-explanatory. The mild steel support is shaped like a microtome blade with a 20°-angle. A wedge-shaped thickening with a 10°-angle is left in the middle and bears two small pegs which fit into the slots in the blade. When the blade is in position and held firmly by the facing piece applied to its surface, it will be inclined at an angle of 30°. With a facet angle of 50° on the blade, this will allow for a clearance of 5° ($30^\circ - \frac{50^\circ}{2}$) between the block and the surface of the facet.

I am indebted to Mr. James Higgins for his help and skill in making holders and carriers for the blades as described above.

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The Use of the Philips 'Mirror-condenser Lamp' in Microscopy

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SUMMARY

The primary source of light is a coiled filament. Ellipsoidal and spherical mirrors ollect nearly all the light it produces and project it on to an area of about $4\frac{1}{2}\times 3\frac{1}{2}$ mm utside the bulb. One end of a solid glass rod is placed in or near this area. The ght travels along the rod by internal reflexions. A bull's-eye lens, placed beyond the ther end of the glass rod, focuses the emerging light on the lower focal plane of the ubstage condenser of the microscope. Very intense illumination is produced. It is moderated by a variable resistor.

The lamp is suitable for use with objectives of focal lengths from 16 mm downwards. t is particularly adapted to high-power microscopy of all sorts. It is easy to use.

THE purpose of this paper is to show how the new Philips 'mirror-condenser lamp' (Alphen and Bierman, 1958), which is intended for use in cinematography, can serve as a very intense and convenient source of light n microscopy.

The 'lamp' or bulb consists of a filament inside a glass container of special hape (see fig. 1). The filament consumes 50 watts (8 volts, 6·25 amps). The ulb is constructed in such a way that nearly all the rays emitted by the lament are concentrated into a roughly rectangular area measuring about $\frac{1}{2}\times 3\frac{1}{2}$ mm.

The inside of the bulb is silvered, except over a circular area of clear glass, bout 17 mm in diameter. The side on which the clear glass area is situated ill be called the front of the bulb. This front side of the bulb is hemispherical. The opposite or posterior side of the bulb is ellipsoidal. The filament—a ingle spiral wire—is placed in the primary focus of the ellipsoidal mirror. The light that reaches this mirror from the filament forms an image of the atter at the secondary focus, outside the bulb, about 11 mm in front of the lear glass. The light that strikes the silvered part of the hemispherical front of the bulb is reflected back to the ellipsoidal mirror and comes to a focus in he same place as the light that passes directly from the filament to the ellipsoidal mirror.

Only the central point of the filament is accurately imaged at the secondary ocus. The image formed by the rest of the filament suffers from coma. Since he filament is spiral, it does not lie wholly in one plane, and this also interferes with the formation of an accurate image at the secondary focus. The mage is therefore blurred, and a nearly evenly illuminated area results.

An intense, fairly large, evenly illuminated source of light is ideal for

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microscopy. It was therefore decided to try to use the mirror-condenser bull in Köhler illumination. A bull's-eye lens was set in front of the image cast b the bulb in such a position that an image of the blurred image at the secondar focus was thrown on to the lower focal plane of the substage condenser of th microscope. It was found, however, that the bulb could not be used in thi way. The field of view was unevenly illuminated, for the following reason An image of the filament itself (not of its blurred image) was thrown by th bull's-eye on to a plane situated between the latter and the mirror of the micro scope, and this image was picked up by the substage condenser and reproduce (out of focus) in the plane of the object on the stage of the microscope.

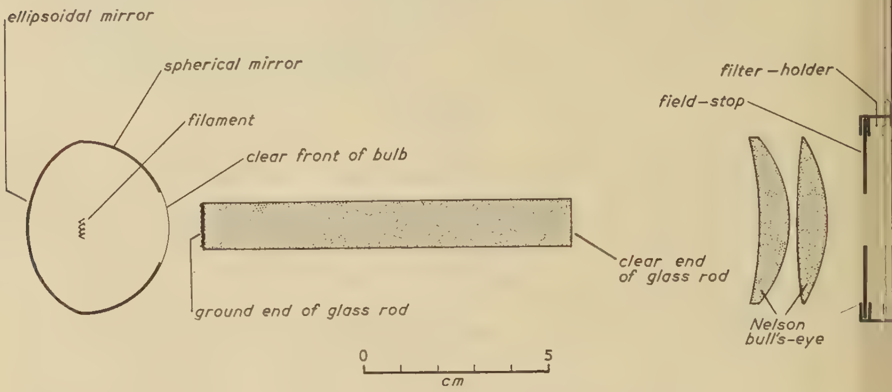

FIG. 1. Diagram of the new lamp (accurately to scale).

In order to get rid of this unwanted image of the filament, use was made o the internal reflexions of a solid, cylindrical glass rod, as suggested by Gordo (1908) and Welch (1930). The rod, 12½ mm in diameter and 10 cm long, wa put in front of the lamp, in such a position that one end of it was in or nea the plane of the secondary focus of the ellipsoidal mirror, while its axis wa in a direct line with the centre of the filament. It was found best to grind th end of the rod that was placed in or near the focus of the ellipsoidal mirror while the other end was left clear. It would be possible to focus the clear end o the glass rod directly on the object, by means of the substage condenser of th microscope, but this was found to be inconvenient. The field of view produce in this way was too small for low-power objectives, and was not quite evenl illuminated. Also, a lot of light was scattered too widely to be received by th substage condenser. For these reasons a bull's-eye was placed in front of th clear-glass end of the rod, in such a position that an image of this end wa thrown at or near the lower focal plane of the substage condenser. Thu Köhler illumination was established, the clear end of the glass rod serving a the effective source of light. A Nelson aplanatic bull's-eye of diameter 25 mm and focal length 45 mm was chosen, though a simple plano-convex lens coul be used instead. It was found convenient to place the whole illuminatin system in such a position that the distance from the front lens of the bull's-ey the lower focal plane of the substage condenser, by way of the mirror, was out 20 cm. Field-stops of convenient sizes can be placed in a slot in front the bull's-eye, or an iris diaphragm may be used instead. A filter-holder y be placed in front of the field-stop or between the bull's-eye and the ar end of the glass rod.

An advantage over the Pointolite and other bulbs commonly used for high-wer microscopy is the relatively large size of the illuminated area that is aged by the bull's-eye on the lower focal plane of the substage condenser. so, there is no obstruction between this area and the bull's-eye, and the ter can therefore have any desired aperture and focal length. The glass bulb the Pointolite is large, and a bull's-eye cannot be brought close to the tung-en ball or balls. In the case of the Pointolite bulb adapted to alternating rrent, the minimum distance is 37 mm. Bull's-eyes of short focus and large erture can therefore not be used. Most of the light emitted by the tungsten ll or balls is wasted.

A comparison was made between the intensity of the light available from Pointolite and that available from the new lamp described in the present per. The Pointolite chosen for the comparison was the Baker-Longworth mp (Baker, 1956), which embodies a two-ball 150 c.p. bulb working on ternating current. A field-stop 14 mm in diameter was placed in front of the ll's-eye lens of each lamp, and these lenses were both focused to give a rilliantly illuminated circle 3 cm in diameter. (Since highly corrected optical stems were not used, the brilliant circle was in each case surrounded by an ea of less intense illumination; this was particularly marked with the new mp.) This circle of brilliant illumination would suffice to cover the aperture f any ordinary substage condenser. The intensity of the light in the two circles as measured with a Weston 'Lightometer', model E 703. To render this eter capable of measuring very bright light, the effective aperture of its hotosensitive surface was reduced by the use of a stop. In the circle illumi-ated by the Pointolite the intensity of the light was 360 foot-candles; in that luminated by the new lamp, 588 foot-candles. It must be mentioned, how-ver, that to produce a circle of bright light 3 cm in diameter, it was necessary place the Pointolite further away from the circle than the new lamp, and the eld-step therefore subtended a smaller angle at any point in the bright circle han did the field-stop of the new lamp.

The whole illuminating system (bulb, glass rod, bull's-eye, field-stop, and lter-holder) can easily be mounted on a board measuring $26 \times 6\frac{1}{2}$ cm, and this an be placed in the grip of an ordinary retort-stand. The bulb is pre-centred in ts holder, and a new one can therefore be put in its correct position without ny need for re-centring. There is so little stray light that no elaborate shield-ng is required. The bulb itself may be freely exposed to the air, and this revents overheating.

It is best to fix the position of the bulb and bull's-eye permanently, but to ount the glass rod in such a way that it can be moved to and fro through a istance of about 1 cm in the direction of its axis. In focusing the lamp one should slide the rod until the image of the clear end of it is thrown on th lower focal plane of the substage condenser of the microscope. In practice suffices to regard the substage filter-holder of the microscope as lying in th lower focal plane of the condenser. A circular piece of white cardboard place in this filter-holder makes it easy to focus and aim the light (Galbraith, 1955 The exact position of the ground end of the glass rod in relation to the secondary focus of the ellipsoidal mirror is of no importance. The filament ca equally well be focused on the ground glass, or a few mm short of it, or a fe mm beyond it (within the glass rod).

It is necessary to use a variable resistor to moderate the intensity of th light. We have used a Curtis 100-watt rotary rheostat in conjunction with G.E.C. transformer no. XT. 5409 (240 to 8 volts). Provided that the filamen is always heated gradually, the bulb seems to last indefinitely, though its lif is said to be short if the current is switched on directly without the use of variable resistor.

The new lamp gives good illumination with objectives of focal lengths from 16 mm downwards, without change of bull's-eye. It is particularly adapte to high-power visual and photographic work in phase-contrast, interference and dark-ground microscopy.

The bulb, made by Philips Electrical, is obtainable through all camera shops; the pre-focusing bulb-holder (P 15S/19, with round base) from Carpenter and Richardson, Paduoc House, Beresford Avenue, Wembley Middlesex.

We thank Mr. F. K. Filipowski for much skilful practical assistance.

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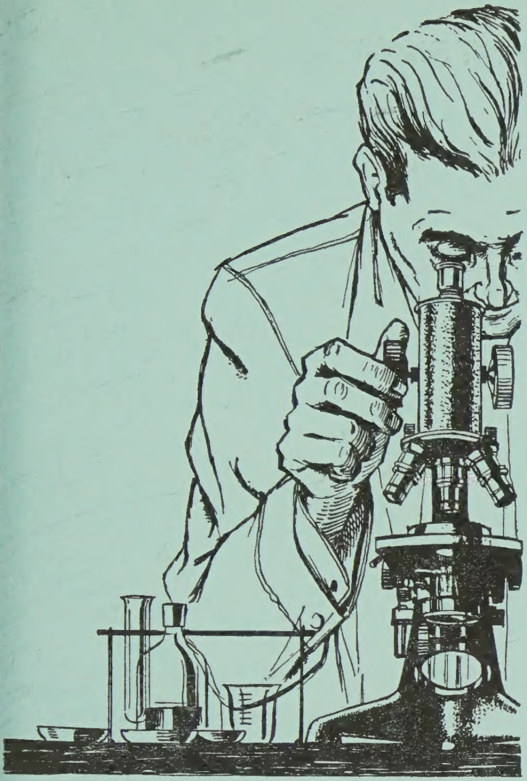

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CONTENTS

Bone, Q. Observations upon the Nervous Systems of Pelagic Tunicates 165

Burnstock, G. The Morphology of the Gut of the Brown Trout (*Salmo trutta*) 183

Burnstock, G. The Innervation of the Gut of the Brown Trout (*Salmo trutta*) 199

Davey, K. G. Spermatophore Production in *Rhodnius prolixus* 221

Przełęcka, A. A Study of Rawitz's 'Inversion Staining' 233

Dasgupta, B. The Feulgen Reaction in the Different Stages of the Life-cycles of Certain Sporozoa 241

Matty, A. J., and **Matty, J. M.** A Histochemical Investigation of the Pituitary Glands of some Teleost Fish 255

Clayton, Blanche-P. The Action of Fixatives on the Unmasking of Lipid 265

Chayen, J., and **Gahan, P. B.** An Improved and Rapid Embedding Method 277

Chayen, J., Gahan, P. B., and **La Cour, L. F.** The Nature of a Chromosomal Phospholipid 279

Wigglesworth, V. B. The Histology of the Nervous System of an Insect, *Rhodnius prolixus* (Hemiptera). I. The Peripheral Nervous System 285

Wigglesworth, V. B. The Histology of the Nervous System of an Insect, *Rhodnius prolixus* (Hemiptera). II. The Central Ganglia 299

Wigglesworth, V. B. A Simple Method for Cutting Sections in the 0·5 to 1μ Range, and for Sections of Chitin 315

Baker, J. R., and **Llowarch, W.** The Use of the Philips 'Mirror-condenser Lamp' in Microscopy 321

NOTICE TO CONTRIBUTORS inside front cover

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